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(57) Abstract

The present invention relates to a method of enhancing an immune response to a pathogen in a host using intranasal administration of interleukin-12 (IL-12). In one embodiment, the present invention relates to a method of inducing an immune response to a pathogen in a host, which comprises administering intranasally to the host an effective amount of IL-12 and an antigen of the pathogen. In another embodiment, the present invention relates to a method of enhancing an immune response to a pathogen in a host, which comprises administering intranasally to the host an effective amount of IL-12 and an antigen of the pathogen. In a particular embodiment, the present invention relates to a method of inducing an immune response to a mucosal pathogen in a host, which comprises administering intranasally to the host an effective amount of IL-12 and an antigen of the pathogen. Also encompassed by the present invention is a method of inducing a Th1-like immune response to a pathogen in a host, comprising administering intranasally to the host an effective amount of IL-12 and an antigen of the pathogen. The present invention also relates to a method of enhancing a mucosal immune response to a pathogen in a host, which comprises administering intranasally to the host an effective amount of IL-12 and an antigen of the pathogen.

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ENHANCEMENT OF IMMUNITY BY INTRANASAL INOCULATION OF IL-12

RELATED APPLICATION(S)

This application is a Continuation-in-Part of U.S. Application No. 09/035,188 filed March 5, 1998, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Mucosal surfaces are the major portals of entry for bacteria and viruses, and therefore constitute the first line of defense for the host. As such, immunization strategies that enhance mucosal immunity have practical significance for preventing infectious disease. Most parenterally administered vaccines, however, are only partially effective at inducing optimal mucosal immunity. Thus, adjuvants that can enhance mucosal immunity and be delivered in a safe, non-invasive manner are needed.

SUMMARY OF THE INVENTION

As described herein, intranasal interleukin-12 (IL-12) treatment can effectively enhance antigen-specific immune responses and enhance immunization strategies for mucosal vaccines. Thus, the present invention relates to methods of enhancing and/or inducing an immune response (e.g., systemic, mucosal) to a pathogen in a host (e.g., mammalian, including human), which comprises administering intranasally (i.n.) to the host an effective amount of IL-12 and an antigen (e.g., a protein, carbohydrate, lipid, recombinant DNA, whole organism, toxin, organic molecule) of the pathogen. As described herein, the immune response can be antigen-specific. In addition, the immune response can result in enhanced expression of a Th1-type cytokine response (e.g., expression of interferon-γ) and/or a humoral response (e.g., IgG2a, IgG2b, IgG3).

In one embodiment, the present invention relates to a method of enhancing an immune response to a pathogen in a host, which comprises administering i.n. to

the host an effective amount of IL-12 and an antigen of the pathogen. In another embodiment, the present invention relates to a method of inducing an immune response to a pathogen in a host, which comprises administering i.n. to the host an effective amount of IL-12 and an antigen of the pathogen.

In a particular embodiment, the present invention relates to a method of inducing an immune response to a mucosal pathogen in a host, which comprises administering i.n. to the host an effective amount of IL-12 and an antigen of the pathogen.

Also encompassed by the present invention is a method of inducing a Th1-like immune response to a pathogen in a host, comprising administering i.n. to the host an effective amount of IL-12 and an antigen of the pathogen.

The present invention also relates to a method of enhancing a mucosal immune response to a pathogen in a host, which comprises administering i.n. to the host an effective amount of IL-12 and an antigen of the pathogen.

The finding that IL-12 administered i.n. is effective for augmenting antigen specific-responses in both mucosal and systemic compartments as described herein, demonstrates that i.n. administration of IL-12 can be used to obtain a potent vaccine adjuvant effect in immunization strategies against pathogens, such as mucosal pathogens.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-1C are bar graphs showing the effects of IL-12 administered intranasally (i.n.) on respiratory mucosal immune responses; the data are presented as average optical density (O.D.) +/- SEM with four mice per group; bronchoalveolar lavage (BAL) were tested at dilutions corresponding to the linear portions of the titration curve (1:64 for IgG1 and IgA, 1:8 for IgG2a).

Figure 2A-2E are graphs of reciprocal serum dilution versus O.D. 405 nm showing the effects of IL-12 administered i.n. on systemic antibody responses; solid symbols represent animals injected with DNP-OVA plus IL-12 and open symbols represent animals injected with DNP-OVA plus phosphate buffered saline (PBS); each line represents binding of antibody from an individual mouse.

Figures 3A-3B are bar graphs showing the effects of IL-12 administered i.n. on total Ig levels; the data are presented as average O.D. +/- SEM with four mice per

group; sera were tested at dilutions corresponding to the linear portions of the titration curve (1:6400 for IgG1, 1:200 for IgG2a).

Figures 4A-4F are bar graphs showing the effects of parenteral (i.p.) and i.n. administration of IL-12 on fecal mucosal responses; the data shown represent day 21 antibody responses for IgA and day 28 responses for IgG isotypes, the peak of each reactive response; the data are presented as average O.D. +/- SEM with 3-4 mice per group.

Figures 5A-5B are graphs of reciprocal serum dilution versus O.D. 405 nm showing the effects of IL-12 administered i.n. on systemic antibody responses; mice were immunized i.n. on day 0 with purified hemagglutinin and neuraminidase derived from influenza virus (HANA) and treated i.n. with either IL-12 (closed triangles) or phosphate buffered saline (PBS) vehicle (open circles) on days 0, 1, 2 and 3; serum anti-HANA antibody levels on day 14 were determined by isotype-specific ELISA using HANA-coated microtiter plates; each line represents binding of antibody from an individual mouse.

Figures 6A-6B are graphs of reciprocal serum dilution versus O.D. 405 nm showing the effects of IL-12 administered i.n. on systemic antibody responses; mice were immunized i.n. on day 0 with HANA and treated i.n. with either IL-12 (closed triangles) or PBS vehicle (open circles) on days 0, 1, 2 and 3 and boosted on day 14; serum anti-HANA antibody levels on day 28 were determined by isotype-specific ELISA using HANA-coated microtiter plates; each line represents binding of antibody from an individual mouse.

Figure 7A-7B are graphs of reciprocal serum dilution versus O.D. 405 nm showing the effects of IL-12 administered i.n. on respiratory mucosal responses; mice were immunized on day 0 with HANA and treated with either IL-12 or PBS vehicle on days 0, 1, 2 and 3 and boosted on days 14 and 28; on day 28 the mice also received IL-12 or vehicle; mice were sacrificed on day 35, and BAL fluid was assayed for anti-HANA antibody levels by ELISA using HANA coated microtiter plates; each line represents binding of antibody from an individual mouse.

Figures 8A-8B are graphic representations showing the effects of IL-12 administered i.n. on early systemic antibody responses to the subunit influenza vaccine. Mice were immunized i.n. on day 0 with H1N1 subunit influenza vaccine, and treated i.n. with either IL-12 (closed triangles) or PBS vehicle (open circles) on

days 0, 1, 2 and 3. Serum anti-H1N1 antibody levels on day 14 were determined by isotype-specific ELISA using HIN1-coated microtiter plates. Each line represents binding of antibody from an individual mouse (4 mice per group). The difference in binding between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle was significant at $p < 0.05$ for IgG2a.

Figures 9A-9E are graphic representations showing the effects of IL-12 administered i.n. on late systemic antibody responses to the subunit influenza vaccine. Mice were immunized i.n. on day 0 with H1N1 subunit influenza vaccine, treated i.n. with either IL-12 (closed triangles) or PBS vehicle (open circles) on days 0, 1, 2 and 3, and boosted with vaccine on days 14 and 28. On day 28, the mice received a second treatment with IL-12 or vehicle. Serum anti-H1N1 antibody levels on day 35 were determined by isotype-specific ELISA using H1N1-coated microtiter plates. Each line represents binding of antibody from an individual mouse (4 mice per group). The differences in binding between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle were significant at $p > 0.05$ for IgG2a, total Ab and total Ig.

Figures 10A-10D are graphic representations showing the effects of IL-12 administered i.n. on respiratory mucosal responses. Mice were immunized i.n. on day 0 with H1N1 subunit influenza vaccine, treated i.n. with either IL-12 or PBS vehicle on days 0, 1, 2 and 3, and boosted with vaccine on days 14 and 28. On day 28, the mice received a second treatment with IL-12 or vehicle. Mice were sacrificed on day 35 and BAL fluid was assayed for anti-H1N1 antibody levels by ELISA using H1N1-coated microtiter plates. Each line represents binding of antibody from an individual mouse (4 mice per group). The differences in binding between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle were significant at $p < 0.05$ for total Ab, IgG1, IgG2a and IgA.

Figures 11A-11D are graphic representations showing that co-administration of influenza subunit vaccine plus IL-12 protects mice from a subsequent influenza virus infection. Mice were immunized i.n. with H1N1 subunit vaccine plus IL-12 (closed triangles), vaccine plus PBS vehicle (open circles), IL-12 only (open diamonds) or PBS vehicle only (open squares). All mice (8 per group) were then challenged i.n. 4-5 weeks later with 10^3 pfu *(A)* or 2×10^3 pfu *(B)* of A/PR/8/34 influenza virus. The mice were monitored daily for mortality and weight loss. The

differences in survival between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS were significant at $p < 0.05$.

Figures 12A-12B are graphic representations showing that IL-12 induced protection against influenza virus infection is mediated by B cells. μMT mice were immunized i.n. on with H1N1 subunit vaccine plus IL-12 (closed triangles), vaccine plus PBS vehicle (open circles) or PBS vehicle only (open diamonds). Wild type (WT) mice were pre-treated with PBS vehicle (open squares). All mice (8 per group) were then challenged i.n. 6-7 weeks later with 10^3 pfu of A/PR/8/34 influenza virus. The mice were monitored daily for mortality and weight loss.

Figure 13 is a graphic representation showing passive transfer of serum from mice immunized with the subunit influenza vaccine plus IL-12 confers protection against influenza virus challenge. Sera were collected from mice immunized with the H1N1 subunit influenza vaccine plus IL-12 (closed triangles), vaccine plus PBS (open circles) or PBS vehicle only (open squares). Pooled serum was diluted 1:10 in sterile PBS and injected i.p. at a dose of 0.1 ml/mouse. All mice (7-8 per group) were then challenged i.n. 5 hours later with 10^3 pfu of A/PR/8/34 influenza virus. The differences in survival between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle were significant at $p < 0.05$.

Figures 14A-14B are graphic representations showing passive transfer of BAL fluid i.n. from mice immunized with the subunit influenza vaccine plus IL-12 confers protection against influenza virus challenge. BAL fluids were collected from mice immunized with the H1N1 subunit influenza vaccine plus IL-12 (closed triangles), vaccine plus PBS (open circles) or PBS vehicle only (open squares). All mice (8 per group) were then inoculated i.n. with pooled BAL fluid and 2×10^3 pfu of A/PR/8/34 influenza virus. The differences in survival between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS were significant at $p < 0.05$.

DETAILED DESCRIPTION OF THE INVENTION

As described herein, systemic and mucosal cytokine and antibody production in mice immunized with a hapten-carrier antigen has been examined. The results show that IL-12 administered i.n. induces Th1-like cytokine and antibody patterns in both spleens and lungs of treated mice. The findings demonstrate that i.n.

inoculation of IL-12 is a powerful means to influence both mucosal and systemic immunity.

Thus, the present invention relates to methods of enhancing and/or inducing immunity to a pathogen (one or more) in a host, which comprises administering i.n. to the host an effective amount of IL-12 and an antigen of the pathogen (e.g., a mucosal pathogen). The methods of the present invention can be used to enhance an immune response to an antigen in a mammalian host, such as a primate (e.g., human), murine, feline, canine, bovine or porcine host.

As used herein, the terms "enhance" and/or "enhancing" refer to the strengthening (augmenting) of an existing immune response to a pathogen. The term also refers to the initiation of (initiating, inducing) an immune response to a pathogen.

An antigen (one or more) for use in the methods of the present invention includes (or can be obtained from), but is not limited to, proteins or fragments thereof (e.g., proteolytic fragments), peptides (e.g., synthetic peptides, polypeptides), glycoproteins, carbohydrates (e.g., polysaccharides), lipids, glycolipids, hapten conjugates, recombinant DNA, whole organisms (killed or attenuated) or portions thereof, toxins and toxoids (e.g., tetanus, diphtheria, cholera) and/or organic molecules. Particular examples of antigens for use in the present invention include hemagglutinin and neuraminidase obtained or derived from the influenza virus.

The antigen can be obtained or derived from a variety of pathogens or organisms, such as bacteria (e.g., bacillus, Group B streptococcus, Bordetella, Listeria, Bacillus anthracis, *S. pneumoniae*, *N. meningiditis*, *H. influenza*), viruses (e.g., hepatitis, measles, poliovirus, human immunodeficiency virus, influenza virus, parainfluenza virus, respiratory syncytial virus), mycobacteria (*M. tuberculosis*), parasites (Leishmania, Schistosomes, Tranpanosomes, toxoplasma, pneumocystis) and fungi (e.g., Candida, Cryptococcus, Coccidiodes, Aspergillus), against which an immune response is desired in a host. The antigen of a pathogen can be obtained using skills known in the art. For example, the antigen can be isolated (purified, essentially pure) directly from the pathogen, derived using chemical synthesis or obtained using recombinant methodology. In addition, the antigen can be obtained from commercial sources. A suitable antigen for use in the present invention is one that includes at least one B and/or T cell epitope (e.g., T helper cell or cytolytic T

cell epitope). Other suitable antigens useful in the compositions of the present invention can be determined by those of skill in the art.

IL-12 is a recently characterized heterodimeric cytokine that has a molecular weight of 75 kDa and is composed of disulfide-bonded 40 kDa and 35 kDa subunits. It is produced by antigen presenting cells such as macrophages and dendritic cells, and binds to receptors on activated T, B and NK cells (Desai, B.B., *et al., J. Immunol., 148*:3125-3132 (1992); Vogel, L.A., *et al., Int. Immunol., 8*:1955-1962 (1996)). It has several effects including 1) enhanced proliferation of T cells and NK cells, 2) increased cytolytic activities of T cells, NK cells, and macrophages, 3) induction of IFN-γ production and to a lesser extent, TNF-α and GM-CSF, and 4) activation of Th1 cells (Trinchieri, G., *et al., Blood, 84*:4008-4027 (1994). IL-12 has been shown to be an important costimulator of proliferation in Th1 clones (Kennedy *et al., Eur. J. Immunol. 24*:2271-2278, 1994) and leads to increased production of IgG2a antibodies in serum when administered i.p. (Morris, S.C., *et al., J. Immunol. 152*:1047-1056 (1994); Germann, T.M., *et al., Eur. J. Immunol., 25*:823-829 (1995); Sher, A., *et al., Ann. N.Y. Acad. Sci., 795*:202-207 (1996); Buchanan, J.M., *et al., Int. Imm., 7*:1519-1528 (1995); Metzger, D.W., *et al., Eur. J. Immunol., 27*:1958-1965 (1997)). Administration of IL-12 i.p. can also temporarily decrease production of IgG1 antibodies (Morris, S.C., *et al., J. Immunol. 152*:1047-1056 (1994); McKnight, A.J., *J. Immunol. 152*:2172-2179 (1994); Buchanan, J.M., *et al., Int. Imm., 7*:1519-1528 (1995)), indicating suppression of the Th2 response. The purification and cloning of IL-12 are disclosed in PCT publication nos. WO 92/05256 and WO 90/05147, and in European patent publication no. 322,827 (identified as "CLMF").

As used herein, "interleukin-12" and "IL-12" refer to interleukin 12 protein, its individual subunits, multimers of its individual subunits, functional fragments of IL-12, and functional equivalents and/or analogues of "interleukin-12" and "IL-12". As defined herein, functional fragments of IL-12 are fragments which, when administered i.n., modulate an immune response against an antigen in a host. As also defined herein, functional fragments or equivalents of "interleukin-12" and "IL-12" include modified IL-12 protein such that the resulting IL-12 product has activity similar to the IL-12 described herein (e.g., the ability to enhance an immune response when administered i.n.). Functional equivalents or fragments of

"interleukin-12" also include nucleic acid sequences (e.g., DNA, RNA) and portions thereof, which encode a protein or peptide having the IL-12 function or activity described herein (e.g., the ability to enhance an immune response when administered i.n.). In addition, the term includes a nucleotide sequence which through the degeneracy of the genetic code encodes a similar peptide gene product as IL-12 and has the IL-12 activity described herein. For example, a functional equivalent of "interleukin-12" and "IL-12" includes a nucleotide sequence which contains a "silent" codon substitution (e.g., substitution of one codon encoding an amino acid for another codon encoding the same amino acid) or an amino acid sequence which contains a "silent" amino acid substitution (e.g., substitution of one acidic amino acid for another acidic amino acid).

IL-12 suitable for use in the methods of the present invention can be obtained from a variety of sources or synthesized using known skills. For example, IL-12 can be purified (isolated, essentially pure) from natural sources (e.g., mammalian, such as human sources), produced by chemical synthesis or produced by recombinant DNA techniques. In addition, the IL-12 for use with the present invention can be obtained from commercial sources.

An effective amount of IL-12 is administered i.n. in the methods of the present invention which is an amount that induces and/or enhances an immune response to an antigen in the host. In particular, "an effective amount of IL-12" is an amount such that when administered i.n. with an antigen to a host, enhances an immune response to the antigen in the host as described herein, relative to the immune response to the antigen in a host when an effective amount of IL-12 is not administered i.n. to the host. That is, an "effective amount" of IL-12 is an amount that, when administered i.n. with an antigen, it enhances an immune response to an antigen in a host as described herein, relative to the immune response to the antigen if IL-12 is not administered i.n. to the host.

The IL-12 and/or the antigen can be administered i.n. as a prophylactic vaccine or a therapeutic vaccine. That is, the IL-12 can be administered either before (to prevent) or after (to treat) the effects of a pathogen which has appeared and/or manifested in a host. Thus, the IL-12 and/or antigen can be administered to a host who either exhibits the disease state caused by a pathogen from which the antigen is obtained or derived, or does not yet exhibit the disease state caused by a

pathogen from which the antigen is obtained or derived. Thus, the IL-12 and/or antigen can be administered to a host either before or after the disease state is manifested in the host and can result in prevention, amelioration, elimination or a delay in the onset of the disease state caused by the pathogen from which the antigen is obtained or derived.

As described herein the IL-12 and the antigen are administered i.n. to a host. Any convenient route of i.n. administration can be used. For example, absorption through epithelial or mucocutaneous linings (e.g., administering the IL-12 and/or antigen using a nasal mist; administering the IL-12 and/or antigen to the eye using an eye dropper wherein the IL-12 and/or antigen drains into the nasal cavity) can be used. In addition, the IL-12 and antigen can be administered together with other components or biologically active agents, such as adjuvants (e.g., alum), pharmaceutically acceptable surfactants (e.g., glycerides), liposomes, excipients (e.g., lactose), carriers, diluents and vehicles. If desired, certain sweetening, flavoring and/or coloring agents can also be added.

Further, the IL-12 and/or the antigen, in the embodiment wherein the antigen is a protein (peptide), can be administered i.n. by *in vivo* expression of polynucleotides encoding such into a host. For example, the IL-12 or the antigen can be administered to a host using a live vector, wherein the live vector containing IL-12 and/or antigen nucleic acid sequences is administered i.n. under conditions in which the IL-12 and/or antigen are expressed *in vivo*. A host can also be injected i.n. with a vector which encodes and expresses an antigen *in vivo* in combination with IL-12 protein or peptide, or in combination with a vector which encodes and expresses the IL-12 protein *in vivo*. Alternatively, a host can be injected i.n. with a vector which encodes and expresses IL-12 *in vivo* in combination with an antigen in peptide or protein form, or in combination with a vector which encodes and expresses an antigen *in vivo*. A single vector containing the sequences encoding an antigen and the IL-12 protein are also useful in the methods of the present invention.

Several expression vector systems are available commercially or can be reproduced according to recombinant DNA and cell culture techniques. For example, vector systems such as the yeast or vaccinia virus expression systems, or virus vectors can be used in the methods and compositions of the present invention (Kaufman, R.J., *A J. of Meth. in Cell and Molec. Biol.*, *2*:221-236 (1990)). Other

techniques using naked plasmids or DNA, and cloned genes encapsulated in targeted liposomes or in erythrocyte ghosts, can be used to introduce IL-12 polynucleotides into the host (Freidman, T., *Science*, *244*:1275-1281 (1991); Rabinovich, N.R., *et al.*, *Science*, *265*:1401-1404 (1994)). The construction of expression vectors and the transfer of vectors and nucleic acids into various host cells can be accomplished using genetic engineering techniques, as described in manuals like *Molecular Cloning* and *Current Protocols in Molecular Biology*, which are hereby incorporated by reference, or by using commercially available kits (Sambrook, J., *et al.*, *Molecular Cloning*, Cold Spring Harbor Press, 1989; Ausubel, F.M., *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, 1989).

As described herein, i.n. administration of IL-12 and an antigen to a host enhances an immune response in the recipient host. For example, the present invention relates to a method of inducing a Th1-like immune response to a pathogen in a host, comprising administering i.n. to the host an effective amount of IL-12 and an antigen of the pathogen. The present invention also relates to a method of enhancing a mucosal immune response to a pathogen in a host, comprising administering i.n. to the host an effective amount of IL-12 and an antigen of the pathogen. The methods described herein can result in enhanced expression of IFN-γ. In addition, a humoral response can be induced and/or enhanced in a host, which can result in enhanced expression of IgG2a, IgG2b and/or IgG3 antibody. The immune response can be antigen-specific.

In the methods of enhancing an immune response to an antigen in a host, an effective amount of IL-12 is administered i.n. to the host, which is an amount that enhances and/or induces an immune response to the antigen in the host and results in the improved condition of the host (i.e., the disease or disorder caused by the presence of the pathogen from which the antigen is obtained or derived, is prevented, eliminated or diminished). The amount of IL-12 used to enhance an immune response to an antigen in a host will vary depending on a variety of factors, including the size, age, body weight, general health, sex and diet of the host, and the time of administration, duration or particular qualities of the disease state. Suitable dose ranges of IL-12 are generally about 0.5μg to about 150 μg per kg body weight. In one embodiment, the dose range is from about 2.75 μg to about 100 μg per kg

body weight. In another embodiment, the dose range is from about 5 μg to about 50μg per kg body weight. Effective dosages may be extrapolated from dose-response curves derived *in vitro* or in animal model test systems.

In the methods of the present invention, an effective amount of IL-12 is administered i.n. in combination with an antigen. That is, the IL-12 is administered at a time closely related to immunization of the host with an antigen, so that an immune response to the antigen is induced or enhanced in the host relative to the immunization of a host in which IL-12 is not administered. Thus, the IL-12 can be administered i.n. prior to, preferably just prior to, immunization; at the time of immunization (i.e., simultaneously); or after immunization (subsequently). In addition, the IL-12 can be administered i.n. prior to immunization with the antigen followed by subsequent administrations of IL-12 after immunization with the antigen.

As described herein, IL-12 given i.n. and in a non-invasive manner, redirects the mucosal compartment of the immune system toward Th1 type cytokine and antibody profiles. As also described herein, i.n. delivery of IL-12 modulates the patterns of cytokine and antibody expression in distant systemic compartments of the immune system.

Mice immunized i.n. with DNP-OVA plus IL-12 displayed enhanced levels of IFN-γ mRNA in the lungs after 6 hours with maximal expression noted at 24 hours. There was a similar enhancement of IFN-γ mRNA in the spleen after i.n. administration of IL-12. IFN-γ is a potent immunoregulator of Th cell subsets and their effector functions (Trinchieri, G., *et al., Res. Immunol., 146*:423-431 (1995); Trinchieri, G., *Immunol. Today, 14*:335-338 (1993)). Specifically, IFN-γ has been shown to activate macrophages and mediate isotype switching to IgG2a and IgG3 antibody production which is characteristic of Th1-type immune responses (Snapper, C.M., *et al., Science, 236*:944-947 (1987); Snapper, C.M., *et al., J. Immunol., 140*:2121-2127 (1988); Finkelman, F.D., *et al., J. immunol., 140*:1022-1027 (1988)). Similarly, an important negative regulator of T-cell responses is interleukin-10 (IL-10) (Meyaard, L., *et al., J. Immunol., 156*:2776-2782 (1996)). IL-10 is mainly produced by T cells and monocytes and exerts its regulatory effects through its actions on antigen presenting cells (Fiorentino, D.F., *et al., J. Immunol., 146*:3444-3451 (1991); Ding, L., *et al., J. Immunol., 148*:3133-3139 (1992)).

Recently, several investigators have found that IL-12 is able to induce human T cells to secrete IL-10 (Meyaard, L., *et al., J. Immunol., 156*:2776-2782 (1996); Daftarian, P.M., *et al., J. Immunol., 157*:12-20 (1996); Gerosa, F., *et al., J. Exp. Med., 183*:2559-2569 (1996)). In light of these studies, the ability of IL-12 given i.n. to induce IL-10 mRNA in both the lungs and spleens was assessed. The results clearly show the ability of IL-12 to induce IL-10 mRNA. However, maximal expression was only noted at 24 hours post inoculation. The delay in the induction of IL-10 mRNA expression after IL-12 treatment suggests that this cytokine is involved in a feedback mechanism designed to modulate the effects of IL-12/IFN-γ. IL-5 mRNA as a specific marker for Th2 differentiation was also analyzed, and a clear reduction of IL-5 mRNA in the lungs of mice treated with IL-12 was found. The findings are consistent with others (Trinchieri, G., *et al., Res. Immunol., 146*:423-431 (1995); Trinchieri, G., *Immunol. Today, 14*:335-338 (1993); Manetti, R., *et al., J. Exp. Med., 177*:1199-1204 (1993); Hsieh, C.S., *et al., Science, 260*:547-549 (1993)) who examined the effects of IL-12 given i.p. on systemic immunity and adds further support to the immunoregulatory functions of IL-12. The results clearly demonstrate that i.n. IL-12 administration can induce a Th1-type cytokine response in both systemic and mucosal compartments.

Since cytokines that are elaborated *in vivo* can determine the profile of antibodies produced during an immune response (Finkelman, F.D., *et al., Ann. Rev. Immunol., 8*:303-333 (1990)), antigen-specific antibody levels in BAL, sera and fecal extracts were examined. Intranasal delivery of antigen and IL-12 resulted in clear enhancement of BAL IgG2a antibody levels. This is the first evidence that i.n. IL-12 administration can modulate respiratory antibody responses in mice. Yang *et al.* (Yang, Y., *et al., Nature Med., 1*:890-893 (1995)) previously demonstrated that intratracheal inoculation of IL-12 and recombinant adenovirus results in a reduction of antigen-specific IgA in BAL without any alteration in IgG levels. However, the effects of IL-12 in this system were not thoroughly characterized in terms of IgG isotypes and therefore there is little information about the role that IL-12 may play in respiratory antibody responses. Furthermore, the intratracheal route was invasive and not relevant to vaccination protocols. The findings described herein are significant in terms of host defense as protection of the lower respiratory tract against viral infections has been correlated with IgG antibodies (Palladino, G., *et al.,*

J. Virol., 69:2075-2081 (1995)). Furthermore, murine antibodies of the IgG2a isotype are known to be very efficient at opsonization and complement fixation, the primary mechanisms thought to be involved in clearance of respiratory pathogens such as *S. pneumoniae* and *N. meningitidis*.

Previous work showed that IL-12 given i.p. can alter the isotype-restricted antibody response of mice to hen eggwhite lysozyme (HEL) (Buchanan, J.M., *et al., Int. Immunol., 7*:1519-1528 (1995); Metzger, D.W., *et al., Ann. N.Y. Acad. Sci., 795*:100-115 (1996)). Parenteral injections of IL-12 plus HEL greatly elevated HEL-specific serum IgG2a and temporarily suppressed IgG1 antibody production. In addition, others (McKnight, A.J., *et al., J. Immunol., 152*:2172-2179 (1994); Morris, S.C., *et al., J. Immunol., 152*:1047-1056 (1994); Germann, T., *et al., Eur. J. Immunol., 25*:823-829 (1995); Wynn, T.A., *et al., J. Immunol., 157*:4068-4078 (1996); Bliss, J., *et al., J. Immunol., 156*:887-894 (1996)) have demonstrated that i.p. IL-12 administration enhances serum IgG2a, IgG2b and IgG3 antibody responses to protein antigens.

Described herein is the fact that IL-12 delivered i.n. by a non-invasive route is capable of influencing serum antibody responses in a similar manner. Mice that were immunized i.n. with antigen and IL-12 had markedly elevated levels of serum IgG2a, IgG2b and IgG3 compared to animals receiving antigen only. The observed increases in IgG2a and IgG3 levels are consistent with the ability of IL-12 to induce IFN-γ, which is a potent switch factor for both IgG2a and IgG3 antibody responses (Metzger, D.W., *et al., Eur. J. Immunol., 27:*1958-1965 (1997); Snapper, C.M., *et al., Science, 236*:944-947 (1987); Snapper, C.M., *et al., J. Exp. Med., 175*:1367-1371 (1992); Collins, J.T., *et al., Int. Immunol., 5*:885-891 (1993)). In addition, the initial IgG1 suppression seen with IL-12 treatment was lost by day 28, in agreement with previous findings (Buchanan, J.M., *et al., Int. Immunol., 7*:1519-1528 (1995); Metzger, D.W., *et al., Ann. N.Y. Acad. Sci., 795*:100-115 (1996)). These results demonstrate that IL-12 can be delivered i.n. in a non-invasive fashion to influence humoral responses in a manner similar to parenteral administration. Thus, i.n. IL-12 administration would be a safer and effective adjuvant for protein vaccine delivery.

As also described herein, it was found that IL-12 administered i.n. or parenterally resulted in enhancement of fecal IgG2a antibody levels. In contrast, i.n. treatment with IL-12 resulted in reduced IgA expression while parenteral delivery of

IL-12 enhanced IgA levels. These results show an important differential effect of IL-12 given via two different routes of administration. Recently, in contrast to the data described herein, Okada *et al.* (Okada, E., *et al., J. Immunol., 159*:3638-3647 (1997)) reported that i.n. immunization with an HIV DNA vaccine in an IL-12 expressing plasmid did not modify fecal IgA antibody levels. Furthermore, Marinaro *et al.* (Marinaro, M., *et al., J. Exp. Med., 185*:415-427 (1997)) reported that oral delivery of IL-12 in encapsulated liposomes did not alter IgA levels, whereby parenteral administration resulted in reduction of fecal IgA responses. In the Marinaro *et al.* study, mice were immunized orally with antigen for both routes of delivery of IL-12, and, as such, it would be difficult to make a direct comparison with the findings described herein which utilized different routes of delivery of antigen plus IL-12. The results clearly show the ability of IL-12 to differentially affect fecal antibody responses depending on the route of immunization.

There is continued interest in developing safer, more potent and better targeted vaccine adjuvants against a range of infectious diseases (Van Regenmortel, M., *ASM News, 63*:136-139 (1997)). This is in part because the adjuvants currently approved for human use such as alum lack the ability to elicit cell-mediated immunity which is crucial for protection against particular diseases (Gupta, R.K., *et al.*, "The role of adjuvants and delivery systems in modulation of immune response to vaccines In *Novel Strategies in Design and Production of Vaccines*, Eds. Cohen, S. and Shafferman, A., Plenum Press, New York, 1996, pp. 105-113). In the context of vaccine development, the activation of the appropriate Th cells is integral in modulation of the immune response. For example, Th1 type immune responses have been shown to be protective against *Leishmania* (Muller, I., *et al., Immunol. Rev., 112*:95-113 (1989)) and *Listeria* (Kratz, S.S., *et al., J. Immunol., 141*:598-606 (1988)) infections. IL-12 is a key cytokine in immune regulation by its ability to direct Th cells towards a Th1 phenotype with enhancement of IFN-γ secretion and elevation of IgG2a antibody levels. As such, the findings described herein show that the i.n. use of IL-12 as an adjuvant enhances vaccine immunity. Moreover, there are no suitable mucosal adjuvants for clinical use at the current time. An immediate application for IL-12 given by this route would be for use in conjunction with nasal influenza vaccines currently in clinical trials. Since protection against influenza is mediated by IgG antibody (Palladino, G., *et al., J. Virol., 69*:2075-2081 (1995)), co-

administration of IL-12 i.n. would be a means to augment both mucosal and systemic antibody responses towards influenza. In this regard, as shown herein, i.n. administration of a subunit influenza vaccine plus IL-12 markedly enhances systemic and respiratory IgG2a levels.

As described herein a non-invasive i.n. delivery system was used to evaluate the ability of IL-12 to modulate both mucosal and systemic components of the immune system. Mice immunized i.n. with DNP conjugated to OVA (DNP-OVA) in combination with CTB and IL-12 were found to have elevated levels of IFN-γ and IL-10 mRNA transcripts in both lungs and spleens compared to mice not receiving IL-12. In addition, expression of lung IL-5 mRNA was inhibited. Analysis of BAL after IL-12 treatment revealed a significant increase in IgG2a and unaltered IgG1 and IgA anti-OVA antibody levels. Serum IgG2a, IgG2b and IgG3 anti-DNP antibody levels were significantly increased by IL-12 given i.n., while serum IgG1 antibody levels were suppressed, results that are similar to those seen after systemic antigen plus IL-12 administration. Delivery of IL-12 i.n. also enhanced fecal IgG2a and suppressed IgA levels, in contrast to parenteral treatment which increased both fecal IgG2a and IgA antibody expression. These results show that i.n. IL-12 treatment can effectively modulate antigen-specific immune responses and enhance immunization strategies for mucosal vaccines.

In summary, the results clearly demonstrate the effectiveness of IL-12 administered i.n. for augmenting antigen specific-responses in both mucosal and systemic compartments. The findings show that IL-12 can be used as a potent vaccine adjuvant for immunization strategies against mucosal pathogens.

Thus, the methods and described herein can be used to treat and/or prevent a disease or condition associated with a pathogen having one or more antigens in a host. The methods described herein can utilize an effective amount of IL-12 in combination with a single antigen or multiple antigens which can be derived from the same pathogen, from different strains of a pathogen or from different pathogens. Thus, IL-12 and one or more antigens can be used to prevent and/or treat one or more disease or condition associated with the pathogen(s) from which the antigen(s) is derived.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

EXEMPLIFICATION

EXAMPLE 1: MODULATION OF MUCOSAL AND SYSTEMIC IMMUNITY BY INTRANASAL INTERLEUKIN 12 DELIVERY

Materials and Methods

Mice

Six to eight week-old female BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD). Mice were housed in the animal facility at the Medical College of Ohio, and provided food and water *ad libitum*. Animal care and experimental procedures were in compliance with the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Ohio.

INTRANASAL IMMUNIZATION PROTOCOL

Intranasal treatments were performed on mice that had been anesthetized intraperitoneally (i.p.) with a combination of ketamine-HCL (Fort Dodge Laboratories, Fort Dodge, IO) and Xylazine (Bayer Corporation, Shawnee Mission, KA) at concentrations of 80 mg and 16 mg per mouse, respectively. On day 0, mice were immunized i.n. with 50 µl of sterile phosphate-buffered saline (PBS) containing 100 µg of dinitrophenyl hapten conjugated to ovalbumin (DNP-OVA; Biosearch Technologies, San Raphael, CA) and 10 µg cholera toxin B-subunit (CTB; Sigma, St. Louis, MO). This was followed on days 0, 1, 2 and 3 with intranasal i.n. of 1 µg of recombinant murine IL-12 in PBS containing 1% normal BALB/c mouse serum (PBS-NMS) or, in the case of control mice, with PBS-NMS only. Mice were boosted i.n. with the same amount of DNP-OVA and CTB on days 14 and 28. On day 28, the mice also received 1 µg of IL-12 in PBS-NMS or PBS-NMS only. For i.p. inoculations, mice were immunized with 100 µg of DNP-OVA in complete Freund's adjuvant (CFA; Life Technologies, Gaithersburg, MD) on day 0, followed by injection of 1 µg of IL-12 in PBS-NMS on days 0, 1, 2 and 3. Control mice received antigen and PBS-NMS only. Mice were boosted by the same route on days 14 and 28 with DNP-OVA in incomplete Freund's adjuvant (IFA; Life Technologies). On day 28, the mice were also injected i.p. with IL-12 in PBS-NMS or PBS-NMS only. Sera were prepared by bleeding mice from the orbital plexus.

RNA ISOLATION

Total RNA isolation from snap frozen spleens and lungs was performed with Trizol reagent (Gibco-BRL Gaithersburg, MA) according to the manufacturer's instructions. Briefly, the frozen tissues were homogenized with a mortar and pestle, and immediately transferred into polystyrene tubes containing 2.0 ml of Trizol reagent. The homogenized samples were incubated for 5 minutes at room temperature to allow dissociation of the nucleoprotein complexes and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant fluids were mixed for 15 seconds with 0.4 ml of chloroform, incubated for 15 minutes on ice, and centrifuged at 12,000g for 15 minutes at 4°C. Following centrifugation, the RNA in the aqueous phase was precipitated at -20°C for one hour by the addition of 1.0 ml isopropanol. The samples were centrifuged for 15 minutes at 12,000g and the RNA pellet was washed twice with 1.0 ml of 75% ethanol. The pellet was air-dried for 2-5 minutes, solubilized in DEPC-treated water, and stored at -80°C. The concentration of total RNA was calculated using the A260 value for single-stranded RNA (1 A260 unit = 40 μg of single stranded RNA/ml). The final preparation of total RNA yielded a 260/280 ratio of 1.7-2.0.

FIRST STRAND cDNA SYNTHESIS

First strand cDNA synthesis was performed following the manufacturer's instructions (Gibco-BRL). Briefly, 1 μg of oligo(dT), 3 μg of total RNA, and sterile DEPC-treated water were added to a sterile eppendorf tube to a final volume of 11 μl. The mixture was incubated at 70°C for 10 minutes and then chilled on ice. Subsequently, the following components were added in order: 4.0μl of 5X first strand buffer, 2 μl of 0.1 M DDT, and 1 μl of dNTP mixture (10 mM each of dATP, dGTP, dCTP and dTTP). The contents of the tube were mixed gently and incubated at 42°C for 2 minutes, followed by the addition of 1 μl (200 U) of Superscript II reverse transcriptase (RT). The reaction mixture was gently mixed and incubated at 42°C for one hour, then terminated by incubation at 70°C for 15 minutes.

POLYMERASE CHAIN REACTION (PCR)

A 50 μl reaction mixture was prepared in a sterile eppendorf tube with the following components: 31.30 μl DEPC treated water, 10.0 μl of 5 times Tris-HCL

buffer (optimal magnesium and pH were determined for each primer set), 2 µl of cDNA from the first strand synthesis, 2 µl primer (20 µm stock concentration), 5.0 µl of dNTP mix (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 2.5 mM dTTP, pH 8.0) (Invitrogen Corporation), and 0.5 µl (2.5 U) of Taq DNA polymerase (Gibco-BRL). The tubes were placed into the wells of the Perkin Elmer Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, CT), incubated at 95°C for 5 minutes and then subjected to the following amplification profile: 1 minute at 95°C, 1 minute at 56°C and 1 minute at 72°C for a duration of 35 cycles. This followed by an incubation at 72°C for 10 minutes followed by a soak cycle at 4°C. The PCR products were separated on a 2.5% agarose gel and stained with ethidium bromide. The bands were visualized and photographed using UV transillumination. Hypoxanthine phosphoribosyl transferase (HPRT) was used as a housekeeping control to ensure equal loading of RNA in all lanes and a 100 bp DNA ladder (Gibco-BRL) was used as a molecular weight marker.

Primer Sequences

HPRT

5' GTT GGA TAC AGG CCA GAC TTT GTT G 3' (SEQ ID NO: 1)

5' GAT TCA ACT TGC GCT CAT CTT AGG C 3' (SEQ ID NO: 2)

IL-5

5' GAC AAG CAA TGA GAC GAT GAG 3' (SEQ ID NO: 3)

5' GTT ATC CTT GGC TAC ATT ACC 3' (SEQ ID NO: 4)

IL-10

5' ATG CAG GAC TTT AAG GGT TAC TTG GGT T 3' (SEQ ID NO: 5)

5' ATT TCG GAG AGA GGT ACA AAC GAG GTT T 3' (SEQ ID NO: 6)

IFN-γ

5' TGA ACG CTA CAC ACT GCA TCT TGG 3' (SEQ ID NO: 7)

5' CGA CTC CTT TTC CGC TTC CTG AG 3' (SEQ ID NO: 8)

COLLECTION OF BRONCHOALVEOLAR LAVAGE (BAL) AND FECAL EXTRACTS

For collection of BAL, the mice were sacrificed and their tracheas were exposed and intubated using a 0.58 mm OD polyethylene catheter (Becton Dickinson, Sparks, MD). The lungs were lavaged two to three times with PBS containing 5 mM EDTA. Approximately 1.5 ml of lavage fluid was obtained per mouse and blood contamination was monitored using Hemastix (Bayer Corporation, Elkhart, IN). The recovered BAL fluid was centrifuged at 12,000g for 5 minutes at 4°C and the supernatant was stored at -70°C until use. Fecal extracts were prepared by the method of deVos and Dick (deVos, T., *et al., J. Immunol. Meth., 141*:285-288 (1991). Briefly, 0.1 g of fecal material from each mouse was mixed with 1 ml PBS and allowed to incubate for 15 minutes at room temperature. The sample was subsequently vortexed for 5 minutes and centrifuged at 12,000 x g for 10 minutes. The supernatant was then stored at -70°C.

DETECTION OF ANTIBODY AND ISOTYPE LEVELS BY ELISA

Anti-DNP and anti-OVA antibody levels were determined by ELISA as described (Buchanan, J.M., *et al., Int. Immunol., 7*:1519-1528 (1995); Metzger, D.W., *Eur. J. Immunol., 27*:1958-1965 (1997)). Briefly, microtiter plates (Nalge Nunc International, Rochester, NY) were coated overnight with 10 μg/ml DNP-bovine serum albumin (BSA) or 100 μg/ml of OVA in PBS. The plates were washed with PBS containing 0.1% (w/v) gelatin and 0.05% (v/v) Tween 20. Serial dilutions of serum or BAL fluid were then added and the plates were incubated for 2 hours at room temperature. The plates were again washed and incubated for 1 hour with goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The plates were washed and ρ-nitrophenyl phosphatase substrate was added to obtain optimal color development. The plates were read at 405 nm with an ELISA microplate reader (Bio-Tek Instruments, Winooski, VT). To detect IgA, the wells were incubated with goat anti-mouse IgA conjugated to biotin (Sigma, St. Louis, MO), washed, and incubated with alkaline phosphatase conjugated to streptavidin (Biorad, Richmond, CA) before addition of substrate. Total immunoglobulins were measured in the same fashion except that the plates were coated with 10 μg/ml

affinity purified goat anti-mouse Ig (Southern Biotechnology Associates) (Buchanan, J.M., *et al., Int. Immunol., 7*:1519-1528 (1995)). In all cases, appropriate working dilutions and isotype specificities of the secondary antibody conjugates were determined using purified myeloma proteins of known isotypes (Sigma, St. Loius, MO). Furthermore, antigen specificity of the assays was established using plates coated with BSA only. Statistical significance was determined using a two-tailed Student *t*-test. Data were considered statistically significant if p values were <0.05.

RESULTS

Intranasal IL-12 delivery induces a Th1-like response in the lungs and spleens

To determine whether intranasal delivery of antigen plus IL-12 would modulate cytokine mRNA expression in the lungs, mice were immunized with DNP-OVA and CTB +/- IL-12, and levels of cytokine mRNA in the lungs of individual animals were analyzed by RT-PCR after 6 and 24 hours. There was found to be a sharp increase in the expression of IFN-γ mRNA in mice 6 hours after treatment with IL-12 and this expression remained elevated for at least 24 hours compared to immunized mice not exposed to IL-12. There were no differences in IL-10 mRNA expression noted in the lungs of IL-12 treated mice after 6 hours but increased expression was observed 24 hours post inoculation. Since IFN-γ mRNA has been found to downregulate Th2 type cytokines such as IL-5 (Mosmann, T.R., *et al., Annu. Rev. Immunol., 7*:145-173 (1989); Coffman, R.L., *et al., Immunol. Rev., 123*:189-207 (1991)), expression of IL-5 mRNA was also examined and a strong decline by 6 hours, which was still apparent after 24 hours, was observed.

Cytokine expression in the lungs was compared to that in spleens after i.n. inoculation of antigen plus IL-12. There was an enhancement of splenic IFN-γ mRNA expression 6 hours after treatment with IL-12. This increase was still pronounced at 24 hours whereas untreated mice had nearly undetectable levels of IFN-γ mRNA at this time point. Increases of IL-10 mRNA levels were also detected in the spleens of IL-12 treated mice, with maximal expression at 24 hours compared to untreated controls. The ability of IL-12 given i.p. to induce systemic IL-10 expression was previously shown by others (Meyaard, L., *et al., J. Immunol., 156*:2776-2782 (1996); Daftarian, P.M., *et al., J. Immunol., 157*:12-20 (1996);

Gerosa, F., *et al., J. Exp. Med., 183*:255902569 (1996)). Finally, no IL-5 was detected in the spleens of either IL-12 treated or control mice in contrast to the lungs where IL-5 mRNA was detected after i.n. antigen treatment but suppressed by co-administration of IL-12. Simultaneous amplification of HPRT mRNA confirmed that equal amounts of RNA were utilized in all of the RT-PCR reactions. These results clearly demonstrate that i.n. administration of IL-12 can modulate antigen-driven cytokine responses in both mucosal and systemic compartments, resulting in significant enhancement of IFN-γ and IL-10 mRNA expression. These findings also provide strong evidence for the ability of i.n. delivery of IL-12 to downregulate the expression of the Th2-associated cytokine, IL-5.

Intranasal IL-12 administration modulates respiratory antibody responses

Previous work (Buchanan, R.I., *et al., Int. Immunol., 7*:1519-1528 (1995); Metzger, D.W., *et al., Ann. N.Y. Acad. Sci., 795*:100-115 (1996); McKnight, A.J., *et al., J. Immunol., 152*:2172-2179 (1994); Morris, S.C., *et al., J. Immunol., 152*:1047-1056 (1994); Germann, T., *et al., Eur. J. Immunol., 25*:823-829 (1995); Wynn, T.A., *et al., J. Immunol., 157*:4068-4078 (1996); Bliss, J., *et al., J. Immunol., 156*:887-894 (1996)) demonstrated the ability of parenteral delivery of IL-12 to enhance serum IgG2a antibody responses to protein and hapten-carrier antigens. IL-12 also temporarily suppresses IgG1 production (Buchanan, J.M., *et al., Int. Immunol., 7*:1519-1528 (1995); Metzger, D.W., *et al., Ann. N.Y. Acad. Sci., 795*:100-115 (1996)). It has now been found, as described herein, that i.n. administration of IL-12 modulates respiratory antibody responses in a similar fashion. BAL fluids were collected on day 35 of the immune response and analyzed by ELISA. Mice that were immunized with DNP-OVA and treated i.n. with IL-12 showed a dramatic enhancement ($p<0.05$) in IgG2a anti-OVA antibody levels compared to immunized mice not exposed to IL-12 (Figures 1A-1C). There were no differences in IgG1 or IgA anti-OVA antibody levels between control and experimental groups. Blood contamination was ruled out by the absence of albumin in respiratory secretions. These results provide the first evidence for the ability of i.n. delivery of IL-12 to alter a respiratory antibody response.

Intranasal IL-12 administration modulates serum antibody responses

ELISA analyses of day 14 sera revealed that i.n. inoculation of DNP-OVA and IL-12 also caused a significant increase ($p<0.05$) in serum IgG2a anti-DNP antibody levels compared to control mice that received DNP-OVA and vehicle (Figures 2A-2E). In addition, there was significant enhancement ($p<0.05$) of serum IgG2b and IgG3 anti-DNP antibody levels after IL-12 treatment. Importantly, the serum IgG2a, IgG2b and IgG3 anti-DNP responses were still elevated 28 days after i.n. IL-12 treatment. There was also suppression of day 14 serum IgG1 anti-DNP antibody production in IL-12 treated mice but little change in IgA anti-DNP antibody levels. However, the initial IgG1 suppression observed with IL-12 treatment was lost by day 28 of the immune response showing that the suppression of IgG1 was only a temporary effect. The effects of i.n. IL-12 treatment on serum levels of total (nonspecific) IgG1 and IgG2a were also examined. It was found that IL-12 treated mice had a corresponding increase in serum IgG2a and a decrease in IgG1 14 days after treatment (Figures 3A-3B). This pattern was still observed four weeks after IL-12 inoculation.

Influence of IL-12 on fecal antibody

The effects of IL-12 given i.n. or i.p. on fecal antibody responses was examined. Mice that received antigen and IL-12 by either route had significantly higher levels ($p<0.05$) of fecal IgG2a anti-DNP antibody levels compared to immunized mice not exposed to IL-12 (Figures 4A-4F). In fact, mice that received only antigen parenterally had no detectable IgG2a in fecal extracts. While parenteral treatment with antigen and IL-12 also resulted in enhancement of fecal IgA levels ($p<0.05$), i.n. delivery of IL-12 resulted in a decrease ($p<0.05$) of IgA antibody levels. There were no significant differences in fecal IgG1 antibody levels between IL-12 treated and control groups after parenteral or i.n. routes of immunization. These results show that i.n. delivery of IL-12 and antigen induces shifts in IgG production similar to those seen after parenteral injection of IL-12. However, only parenteral administration of IL-12 results in enhanced mucosal IgA antibody levels.

Effects of IL-12 on systemic antibody responses using purified hemaglutinin and neuraminidase derived from influenza virus

The effects of IL-12 administered i.n. on systemic antibody responses were examined using purified hemaglutinin and neuraminidase derived from influenza virus (HANA). Mice were immunized i.n. on day 0 with HANA and treated i.n. with either IL-12 or PBS vehicle on days 0, 1, 2 and 3. Serum anti-HANA antibody levels on day 14 were determined by isotype-specific ELISA using HAHA-coated microtiter plates. See Figures 5A-5B. In addition, mice were immunized i.n. on day 0 with HANA and treated i.n. with either IL-12 or PBS vehicle on days 0, 1, 2 and 3 and boosted on day 14. Serum anti-HANA antibody levels on day 28 were determined by isotype-specific ELISA using HAHA-coated microtiter plates. See Figures 6A-6B.

Effects of IL-12 administered i.n. on respiratory mucosal responses

The effects of IL-12 administered i.n. on respiratory mucosal responses were examined. Mice were immunized on day 0 with HANA and treated with either IL-12 or PBS vehicle on days 0, 1, 2 and 3 and boosted on days 14 and 28; on day 28 the mice also received IL-12 or vehicle. Mice were sacrificed on day 35, and BAL fluid was assayed for anti-HANA antibody levels by ELISA using HANA coated microtiter plates. See Figures 7A-7B.

EXAMPLE 2 INTRANASAL INTERLEUKIN-12 IS A POWERFUL ADJUVANT FOR PROTECTIVE MUCOSAL IMMUNITY

Methods

Mice

Six-to eight-week old female BALB/c mice were obtained from The National Cancer Institute (Bethesda, MD). C57BL/6 IgM deficient (μMT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in the animal facility at the Medical College of Ohio and provided food and water *ad libitum*. All animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

Immunizations

Intranasal treatments were performed on mice that had been anesthetized i.p. with a combination of Ketamine HCL (Fort Dodge Laboratories, Fort Dodge, IO) and Xylazine (Bayer Corporation, Shawnee Mission, KA). Mice were immunized i.n. on day 0 with 25 μl of sterile PBS containing 1 μg of subunit influenza vaccine which consisted of soluble hemagglutinin subtype 1 (H1) and neuraminidase subtype 1 (N1) purified from influenza virus A/PR8/34 (provided by Dr. Doris Bucher, New York Medical College, New York, NY). This was followed on days 0, 1, 2 and 3 with i.n. inoculation of 1 μg of recombinant murine IL-12 in PBS containing 1% normal BALB/c mouse serum (PBS-NMS) or in the case of control mice, with PBS-NMS only. Mice were boosted i.n. with the same amount of vaccine on days 14 and 28. On day 28, the mice also received IL-12 in PBS-NMS or PBS-NMS only. No toxicity was observed with this treatment regimen. Sera were prepared by bleeding mice from the orbital plexus.

RNA Isolation and RT-PCR

Total RNA isolation from snap frozen spleens and lungs was performed with the Ambion Total RNA Isolation Kit (Austin, TX) according to the manufacturer's instructions. Briefly, the frozen tissues were homogenized with a mortar and pestle and immediately transferred into tubes containing 1.0 ml of denaturation solution. Following phenol-chloroform extraction, the homogenized samples were centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatants were subjected to another round of phenol-chloroform extraction and the resulting RNA was precipitated with isopropanol, washed twice with 75% ethanol and solubilized in DEPC-treated water. The concentration of total RNA was determined by spectrophotometric analysis at 260 nm. Three micrograms of total RNA were reverse transcribed into cDNA using a reverse transcription kit (Life Technologies, Gaithersburg, MD) utilizing oligo $(dT)_{16\text{-}18}$ primers. The resulting cDNA was amplified using specific primers for IFN-γ and IL-10 with hypoxanthine phosphoribosyl transferase (HPRT) primers as a control. The sense and antisense primers utilized had the following sequences:

IFN-γ

5'-TGAACGCTACACACTGCATCTTGG-3' (SEQ ID NO: 7) and

5'-CGACTCCTTTTCCGCTTCCTGAG-3' (SEQ ID NO: 8);

IL-10

5'-ATGCAGGACTTTAAGGGTTACTTGGGTT-3' (SEQ ID NO: 5) and

5'-ATTTCGGAGAGAGGTACAAACGAGGTTT-3' (SEQ ID NO: 6);

HPRT

5'-GTTGGATACAGGCCAGACTTTGTTG-3' (SEQ ID NO: 1) and

5'-GATTCAACTTGCGCTCATCTTAGGC-3' (SEQ ID NO: 2).

PCR amplification was performed by mixing 2 μl of cDNA, 0.25 mM dNTPs (Invitrogen Corporation, San Diego, CA), 0.8 μM primer and 2.5 U of *Taq* DNA Polymerase (Life Technologies) in a final volume of 50 μl in 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2 SO_4$, 0.4 mM $MgCl_2$. The mixtures were incubated at 95°C for 5 minutes and then subjected to the following amplification profile: 1 minute at 95°C, 1 minute at 56°C and 1 minute at 72°C for a duration of 35 cycles. This was followed by a final extension for 10 minutes at 72°C. The PCR products were separated on a 2.5% agarose gel, stained with ethidium bromide and visualized by UV transillumination.

Ribonuclease Protection Assay

Cytokine mRNA levels were determined utilizing the RiboQuant multi-probe ribonuclease protection assay system (Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, 10 μg of total RNA was hybridized to a ^{32}P labeled RNA probe overnight at 56°C. The single-stranded nucleic acid was digested with ribonuclease for 45 minutes at 30°C, subjected to phenol-chloroform extraction, and resolved on a 6% denaturing polyacrylamide gel. Transcript levels were quantified on a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Total RNA was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase and relative cytokine mRNA levels were expressed as arbitrary values.

Collection of Bronchoalveolar Lavage Fluid

For collection of BAL fluid, the mice were sacrificed and their tracheas intubated using a 0.58 mm OD polyethylene catheter (Becton Dickinson, Sparks, MD). The lungs were then lavaged two to three times with PBS containing 5 mM EDTA. The recovered BAL fluid was centrifuged at 12,000 x g for 5 minutes at 4°C and the supernatant was stored at -70°C until use.

Detection of Antibody and Isotype Levels by ELISA

Anti-H1N1 levels in serum and BAL were determined by ELISA essentially as described (Buchanan, J.M., *et al., Int. Immunol., 7*:1519-1528 (1995); Buchanan, R.M.,*et al/. J. Immunol., 161*:5525-5533 (1998)) with minor modifications. Briefly, microtiter plates (Nalge Nunc International, Rochester, NY) were coated overnight with 1 μg/ml of H1N1 in PBS. The plates were washed with PBS containing 0.3% Brij-35 (Sigma, St. Louis, MO) and blocked for 1 hour at room temperature with PBS containing 5% fetal calf serum (Hyclone Laboratories, Logan, UT) and 0.1% Brij-35. Serial dilutions of serum were added and the plates incubated for 2 hours at room temperature. The plates were washed and incubated with goat anti-mouse IgG1 or IgG2a conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After incubation for 1 hour, the plates were washed and p-nitrophenyl phosphatase substrate was added to obtain color development. Plates were read at 405 nm with an ELISA microplate reader (Bio-Tek Instruments, Winooski, VT). To detect IgA, the plates were incubated with goat anti-mouse IgA conjugated to biotin (Sigma), then washed and incubated with alkaline phosphatase-conjugated streptavadin (BIO RAD, Richmond, CA). Total immunoglobulins were measured in the same fashion except that the plates were coated with 10 μg/ml affinity-purified goat anti-mouse Ig (Southern Biotechnology Associates) (Buchanan, J.M., *et al., Int. Immunol., 7*:1519-1528 (1995)). In all cases, appropriate working dilutions and isotype specificities of the secondary antibody conjugates were determined using purified myeloma proteins of known isotypes (Sigma). Statistical significance was determined using a two-tailed Student *t*-test. Data were considered statistically significant if p values were <0.05.

Virus Challenge

For protection studies, mice were immunized i.n. on day 0 with 25 μl of PBS containing 1 μg of H1N1 subunit influenza vaccine. This was followed on days 0, 1, 2 and 3 with i.n. inoculation of 1 μg of IL-12 in PBS-NMS or with PBS-NMS only. Some mice received only IL-12 in PBS-NMS or only PBS-NMS (no H1N1 subunit vaccine). Approximately 4-5 weeks after primary immunization, viral challenge was performed using infectious A/PR8/34 influenza virus (provided by Dr. Doris Bucher) administered i.n. to anesthetized mice in 40 μl of sterile PBS. The mice were weighed daily and monitored for morbidity and mortality.

Passive Transfer of Sera and BAL Fluid

For passive transfer experiments, sera were obtained on day 28 after i.n. immunization with the H1N1 subunit vaccine. Mice were injected i.p. with 100 μl of a 1:10 dilution of pooled serum and challenged 5 hours later with infectious influenza virus i.n. BAL fluid collected from mice on day 35 after i.n. immunization with H1N1 subunit influenza vaccine was centrifuged to remove cells and the supernatant was administered i.n. to anesthetized mice together with virus in a total volume of 40 μl.

Results

Intranasal IL-12 Administration Induces Expression of Th1 Type Cytokine Responses in the Lungs and Spleens of Immunized Mice

IL-12 given parenterally has profound regulatory effects on the immune system through its ability to preferentially activate Th1 and NK cells, and induce IFN-γ production (Trinchieri, G., *et al., Res. Immunol., 146:*423-431 (1995); Gately, M.K., *et al., Annu. Rev. Immunol., 16:*495-521 (1998)). As described herein, the effects of i.n. administration of IL-12 on respiratory cytokine gene expression have now been examined. Analysis of cytokine mRNA expression in the lungs of individual mice (3 mice per group) after a single i.n. inoculation of IL-12 or PBS vehicle and H1N1 subunit influenza vaccine. Mice were sacrificed 24 hours or 48 hours after treatment, and total lung RNA was assayed for the expression of the indicated cytokines by RT-PCR: IL-10 (455 bp), IFN-γ (459 bp) and HPRT (162 bp). It was found the i.n. treatment of mice with H1N1 subunit influenza vaccine

and IL-12 had an enhancing effect on expression of lung IFN-γ mRNA levels within 24 hours compared to immunization with vaccine only. This increase in IFN-γ mRNA levels was still evident 48 hours after IL-12 inoculation.

It has been previously demonstrated (Meyaard, L., *et al., J. Immunol., 156:*2776-2782 (1996); Daftarian, P.M., *et al., J. Immunol., 157:*12-20 (1996); Gerosa, F., *et al., J. Exp. Med., 183:*2559-2569 (1996)) that treatment with IL-12 enhances expression of IL-10 mRNA. As described herein, i.n. delivery of H1N1 vaccine plus IL-12 also caused a dramatic increase in lung IL-10 mRNA expression. In contrast, there was an absence of IL-10 mRNA in mice that received vaccine only. IL-10 mRNA expression was still significantly elevated after 48 hours in the lungs of IL-12 treated mice compared to animals that received the vaccine alone. The expression of IL-5 mRNA was also examined and no differences were found after IL-12 treatment.

To determine if local mucosal delivery of IL-12 could modulate a distant systemic compartment, cytokine mRNA patterns in the spleens of immunized mice were examined. Analysis of cytokine mRNA expression in the spleens of individual mice (3 mice per group) after a single i.n. inoculation of IL-12 or PBS vehicle and H1N1 subunit influenza vaccine. Mice were sacrificed 24 hours or 48 hours after treatment, and total splenic RNA was assayed for the expression of the indicated cytokines by RT-PCR: IL-10 (455 bp), IFN-γ (459 bp), and HPRT (162 bp). Intranasal administration of H1N1 subunit vaccine plus IL-12 resulted in a substantial increase in splenic IFN-γ mRNA expression within 24 hours compared to mice that received vaccine alone. Elevated levels of IFN-γ were still evident at 48 hours in IL-12 treated mice. Splenic IL-10 mRNA levels remained elevated at both 24 hours and 48 hours after IL-12 treatment. Finally, no IL-5 mRNA was detected in the spleens of either IL-12 treated or control animals. Simultaneous amplification of HPRT mRNA confirmed that equal amounts of RNA were utilized in all RT-PCR reactions. To further quantify the levels of cytokine mRNA transcripts observed after i.n. immunization with influenza vaccine, cytokine mRNA levels in the lungs and spleens were analyzed by ribonuclease protection assay. It was found that IFN-γ mRNA levels were increased 2-fold in the lungs of animals 24 hours and 48 hours after treatment with H1N1 plus IL-12 compared to mice that received vaccine alone (the Table). Furthermore, IL-10 mRNA expression was

enhanced 5-fold in the lungs after IL-12 treatment. In the spleens of these animals, IFN-γ mRNA was elevated 5-fold at 24 hours and 2-fold at 48 hours after IL-12 treatment. Similarly, splenic IL-10 mRNA levels were increased 8-fold at 24 hours and 5-fold at 48 hours after IL-12 treatment.

Table. IFN-γ and IL-10 mRNA Levels in the Lungs and Spleens of Mice Immunized with Influenza Subunit Vaccine*

Lungs

Time	Cytokine	H1N1 + PBS	H1N1 + IL-12	Fold Increase
24 hours	IFN-γ	380 ± 6.7	830 ± 61	2.2
	IL-10	1.7 ± 0.8	8.7 ± 1.3	5.1
48 hours	IFN-γ	340 ± 110	630 ± 152	1.9
	IL-10	1.6 ± 0.5	8.6 ± 3.0	5.4

Spleens

Time	Cytokine	H1N1 + PBS	H1N1 + IL-12	Fold Increase
24 hours	IFN-γ	410 ± 115	1900 ± 400	4.6
	IL-10	2.9 ± 1.9	22 ± 4.3	7.6
48 hours	IFN-γ	570 ± 109	1000 ± 170	1.8
	IL-10	2.7 ± 1.6	13 ± 5.0	4.8

*Mice were sacrificed 24 hours and 48 hours after i.n. treatment with H1N1 subunit influenza vaccine ± Il-12. Total RNA was isolated and IFN-γ and IL-10 transcript levels were analyzed by multiplex ribonuclease protection assay. Relative RNA levels were quantitated on a phosphorimager and normalized to glyceraldehyde 3-phosphate dehydrogenase. The cytokine mRNA levels are expressed as arbritary units ± SE.

Co-administration of an Intranasal Vaccine Plus IL-12 has Potent Effects on Systemic Antibody Responses

It was previously demonstrated that parenteral administration of IL-12 alters isotype-restricted antibody responses to hen eggwhite lysozyme (Buchanan, J.M., *et al., Int. Immunol., 7:*1519-1528 (1995)). In addition, as described in Example 1, IL-12 delivered i.n. modulates both mucosal and systemic immunity to the DNP hapten. In this example it has been demonstrated that IL-12 delivered i.n. has similar effects on antibody responses to H1N1 influenza vaccine. Fourteen days after immunization with vaccine by itself or together with IL-12, there was little if any, detectable serum IgG1 anti-H1N1 antibody (Figures 8A-8B). In contrast, IgG2a anti-H1N1 antibody levels were markedly enhanced after IL-12 treatment compared to mice that received vaccine alone. Therefore, i.n. IL-12 treatment resulted in early activation of serum IgG2a antibody responses.

Similar analysis were performed on day 35 sera to determine the long-term effects of i.n. IL-12 treatment. At this time point, IL-12-treated mice had 6-fold higher levels of total anti-H1N1 serum antibody than mice immunized with the vaccine alone (Figures 9A-9E). Moreover, there was an increase in total (non-specific) Ig after i.n. IL-12 treatment. IgG2a antibody levels were still dramatically enhanced in mice that received IL-12. Furthermore, IgG1 anti-H1N1 antibodies, evident in both experimental and control groups, were moderately elevated in IL-12 treated mice compared to mice receiving only vaccine, an observation which is consistent with our previous findings (Buchanan, J.M., *et al., Int. Immunol., 7:*1519-1528 (1995)). There was no IgA detected in the sera of any of the mice. The results clearly demonstrate the ability of IL-12 co-administered as an adjuvant and delivered in a non-invasive form to enhance serum antibody levels.

Intranasal IL-12 Delivery Enhances Respiratory Antibody Levels

The antibody responses in BAL fluid from i.n. immunized mice were also assessed. Analysis of BAL fluid collected on day 35 of the immune response revealed that IL-12-treated mice had enhanced mucosal antibody responses to H1N1 subunit influenza vaccine. As a group, i.n. IL-12 treatment resulted in 15-fold increases in total anti-H1N1 respiratory antibody production compared to mice immunized with vaccine alone (Figures 10A-10D). In addition, there was a 13-fold

increase in total nonspecific Ig in the BAL fluid of mice that received H1N1 plus IL-12 i.n. Importantly, animals that were immunized and treated with IL-12 displayed elevated BAL fluid IgA anti-H1N1 antibody levels compared to animals not exposed to IL-12. This result is in stark contrast to the absence of detectable IgA in the circulation of these mice. It was also found that levels of both IgG1 and IgG2a anti-H1N1 antibodies were dramatically enhanced in BAL fluid after IL-12 administration compared to mice that received vaccine alone. These results firmly establish the influence of IL-12 delivered i.n. in augmenting respiratory antibody expression.

IL-12 Administration Increases the Protective Effects of Influenza Subunit Vaccination

The effects of co-administrating IL-12 and H1N1 i.n. on survival and clinical outcome after challenge with influenza virus were also assessed. Mice were immunized i.n. with H1N1 vaccine on day 0 and treated with 1 μg of IL-12 or PBS vehicle on days 0, 1, 2 and 3. Some mice received only IL-12 or PBS vehicle. Four to five weeks after immunization, the mice were inoculated i.n. with infectious A/PR8/34 influenza virus and monitored daily for morbidity and mortality. In the first experiment, a dose of virus was used that allowed 50% survival of mice after exposure to just vaccine (Figures 11A-11B). It was found that inclusion of IL-12 during vaccination resulted in 100% survival and significant reduction in illness, as evidenced by reduced weight loss compared to mice that received vaccine alone. Mice that were pre-treated with IL-12 or PBS-NMS only (no H1N1 subunit vaccine) displayed progressive weight loss and all died within 11 days after virus challenge.

In a second experiment, a larger dose of virus was used for challenge such that vaccination with H1N1 alone afforded little if any significant protection (Figures 11C-11D). In this case, it was found that vaccination with H1N1 and IL-12 resulted in 50% survival after challenge. Recovery from infection in the surviving mice was evidenced by regaining body weight. As expected, animals that received IL-12 or PBS-NMS alone did not survive virus challenge. Hence, co-administration of IL-12 and the H1N1 subunit influenza vaccine i.n. increased the efficacy of the vaccine and conferred significant protection against lethal doses of live influenza virus.

Enhanced Protection Against Influenza Infection after Vaccination with H1N1 Plus IL-12 is Antibody-mediated

To ascertain the role of humoral immunity in protection from influenza virus infection, the responses of μMT mice, which lack B cells to IL-12 treatment was examined (Kitamura, D., *et al., Nature, 350:*423-426 (1991)). It was found that all μMT mice pre-treated with PBS alone, vaccine alone or vaccine plus IL-12 succumbed to infection by day 10 (Figures 12A-12B). Wild-type mice pre-treated with PBS alone died twelve days after infection. In addition, all mice displayed a steady, progressive loss of body weight. Thus, the enhanced protection conferred by IL-12 treatment is a result of augmented B cell function.

To further determine if protection against influenza virus observed in mice inoculated i.n. with vaccine and IL-12 was mediated by antibody, we transferred pooled serum from these mice into naive animals, which were then challenged with A/PR8/34 influenza virus 5 hours later. Of the animals that received serum from mice inoculated with vaccine or PBS-NMS only, all succumbed to infection (Figure 13). However, animals that received serum from mice immunized with the vaccine plus IL-12 exhibited 50% survival after viral challenge.

Whether antibodies generated in the respiratory secretions of immunized mice played a crucial role in protection against influenza virus infection was also determined. BAL fluid recovered from unvaccinated animals or animals immunized with H1N1 ± IL-12 was administered i.n. to naive mice together with live virus. The results showed that virus challenge together with passive transfer of BAL fluid from mice that were treated with PBS-NMS alone resulted in 100% death by day 7 (Figures 14A-14B). Virus challenge in the presence of BAL fluid from mice immunized with H1N1 alone resulted in survival of only one of 8 infected mice. However, 100% of the animals that received BAL fluid from mice treated with H1N1 plus IL-12 were protected against virus infection. These mice exhibited no transient weight loss over the course of the infection while both of the other treatment groups displayed progressive weight loss leading to death. Furthermore, mice that received BAL fluid from animals immunized with vaccine alone had viral lung titers of 10^3 pfu on day 4 after infection while mice that received BAL fluid from animals treated with vaccine plus IL-12 had viral lung titers of <100 pfu. Finally, the overall health of virus-challenged animals that received BAL fluid from

mice vaccinated with H1N1 plus IL-12 remained noticeably better than mice which received BAL fluid from animals vaccinated with H1N1 alone. Thus, passive transfer of BAL fluid i.n. from mice immunized with H1N1 subunit vaccine plus IL-12 provided dramatic protection against influenza virus challenge.

Discussion

As described herein, IL-12 delivered i.n. with an influenza subunit vaccine serves as a potent mucosal adjuvant and confers increased protection against subsequent viral infection. Use of B cell deficient mice and passive transfer of serum or BAL fluid demonstrated that the protection induced by IL-12 is mediated by antibody.

Analysis of cytokine mRNA production after i.n. treatment of mice with IL-12 revealed an enhancement of IFN-γ mRNA expression in both lungs and spleen within 24 hours. IFN-γ has a variety of immunoregulatory functions, which include induction of the Th1 cell differentiation and activation of NK cells (Boehm, U., *et al., Annu. Rev. Immunol., 15:*749-795 (1998)). In addition, IFN-γ enhances the production of opsonizing murine antibodies such as IgG2a (Buchanan, J.M., *et al., Int. Immunol., 7:*1519-1528 (1995); Metzger, D.W., *et al., Eur. J. Immunol., 27:*1958-1965 (1997); McKnight, A.J., *et al., J. Immunol., 152:*2172-2179 (1994);Wynn, T.A., *et al., J. Immunol., 157:*4068-4078 (1996)). IL-10 mRNA expression was also induced in lungs and spleens by i.n. treatment with IL-12. IL-10 is mainly produced by T cells and monocytes, and has been shown to inhibit Th1 cell differentiation (Fiorentino, D.F., *et al., J. Immunol., 146:*3444-3451 (1991); Ding, L., *et al., J. Immunol., 148:*3133-3139 (1992)). Others (Meyaard, L., *et al., J. Immunol., 156:*2776-2782 (1996); Daftarian, P.M., *et al., J. Immunol., 157:*12-20 (1996); Gerosa, F., *et al., J. Exp. Med., 183:*2559-2569 (1996)) have shown induction of IL-10 after treatment with IL-12, an observation which suggests a feedback mechanism designed to downregulate the inflammatory effects of IL-12 and IFN-γ.

In Example 2, the effects of i.n. IL-12 on responses to a clinically relevant influenza subunit vaccine was examined. IL-12 treatment was found to have a dramatic effect on the early onset of the humoral response, as reflected by significant enhancement of IgG2a anti-H1N1 antibody levels. In comparison, animals that

received vaccine alone did not develop early IgG2a responses. There was little detectable IgG1 antibody during the early phase of the immune response in animals that received vaccine alone or vaccine and IL-12. After 35 days, IgG2a levels were still enhanced in IL-12 treated mice and IgG1 levels were also somewhat elevated, an observation that is in agreement with previous findings in the lysozyme system (Buchanan, J.M., *et al., Int. Immunol., 7:*1519-1528 (1995)). These results demonstrate the long-lasting effects of IL-12 delivered i.n. and provide further evidence for the use of this route of administration for augmenting systemic humoral immunity.

IL-12 i.n. administration also resulted in significant increases in respiratory antibody levels, including IgG and IgA anti-H1N1 antibody levels. IgA is the predominant antibody in mucosal secretions, and is thought to play a major role in preventing attachment of pathogens to mucosal epithelial surfaces (Lamm, M.E., *Annu. Rev. Immunol., 51:*311-340 (1997)).

As also described herein, passive transfer of serum or BAL fluid collected from mice immunized with subunit influenza vaccine and IL-12 resulted in significant protection from morbidity and mortality. The ability of IL-12 to augment antibody levels and enhance protection against influenza virus infection is completely abrogated in μMT mice. The augmented protection conferred by passive transfer of BAL fluid observed here is likely to be the result of significantly enhanced respiratory antibody levels observed after i.n. IL-12 treatment.

Adjuvants that have been used to enhance mucosal immune responses include microbial products such as CT and LT, which have been utilized in a variety of delivery systems (Staats, H.F., *et al., Curr. Opin. Immunol., 6:*572-583 (1994); Elson, C.O., In *Mechanisms in the Pathogenesis of Enteric Disease*, Paul, P.S., *et al.*, eds., (NY:Plenum Press), pages 373-385 (1997)). CT is a potent inducer of the Th2-type responses, whereas LT elicits a mixed Th1 and Th2 response (Marinaro, M., *et al., J. Exp. Med., 185:*415-427 (1997); Takahashi, I., *et al., Infect. Dis., 173:*627-635 (1996)). However, these adjuvants cause severe diarrhea, and are not suitable for use as mucosal adjuvants in humans. There is also a recent report suggesting CT actually suppresses IL-12 production and IL-12 receptor expression (Braun, M., *et al., J. Exp. Med.,* in press (1999)). Furthermore, in respiratory synctial virus lung infections, Th1 responses are protective while Th2 responses

result in lung pathology (Graham, B.S., *et al., J. Clin. Invest., 88:*1026-1033 (1991); Graham, B.S., *et al., J. Immunol., 151:*2032-2040 (1991). The ability of IL-12 administered i.n. to enhance the protective efficacy of an influenza vaccine is therefore of direct relevance for mucosal vaccination protocols.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. A method of inducing an immune response to a pathogen in a host, which comprises administering intranasally to the host an effective amount of interleukin-12 and an antigen of the pathogen.

2. The method of Claim 1 wherein the pathogen is selected from the group consisting of: bacteria, viruses, mycobacteria, parasites and fungi.

3. The method of Claim 2 wherein the bacteria is selected from the group consisting of: *S. pneumoniae*, *N. meningiditis* and *H. influenza*.

4. The method of Claim 2 wherein the virus is selected from the group consisting of: influenza virus, parainfluenza virus, poliovirus and human immunodeficiency virus.

5. The method of Claim 2 wherein the parasite is selected from the group consisting of: Leishmania, Schistosomes, Trapanosomes, toxoplasma and pneumocystis.

6. The method of Claim 1 wherein the antigen is derived from a toxin of the pathogen.

7. The method of Claim 1 wherein the immune response is a Th1-type cytokine response.

8. The method of Claim 7 wherein the Th1-type cytokine response results in enhanced expression of interferon-γ in the host.

9. The method of Claim 1 wherein the immune response is a humoral immune response.

10. The method of Claim 9 wherein the humoral immune response results in enhanced expression of IgG2a, IgG2b and IgG3 antibodies which are specific to the antigen.

11. A method of enhancing an immune response to a pathogen in a host, which comprises administering intranasally to the host an effective amount of interleukin-12 and an antigen of the pathogen.

12. The method of Claim 11 wherein the pathogen is selected from the group consisting of: bacteria, viruses, mycobacteria, parasites and fungi.

13. The method of Claim 12 wherein the bacteria is selected from the group consisting of: *S. pneumoniae*, *N. meningiditis* and *H. influenza*.

14. The method of Claim 12 wherein the virus is selected from the group consisting of: influenza virus, parainfluenza virus, poliovirus and human immunodeficiency virus.

15. The method of Claim 12 wherein the parasite is selected from the group consisting of: Leishmania, Schistosomes, Trapanosomes, toxoplasma and pneumocystis.

16. The method of Claim 11 wherein the antigen is derived from a toxin of the pathogen.

17. The method of Claim 11 wherein the immune response is a Th1-type cytokine response.

18. The method of Claim 17 wherein the Th1-type cytokine response results in enhanced expression of interferon-γ.

19. The method of Claim 11 wherein the immune response is a humoral immune response.

20. The method of Claim 19 wherein the humoral immune response results in enhanced expression of IgG2a, IgG2b and IgG3 antibodies which are specific to the antigen.

21. A method of inducing an immune response to a mucosal pathogen in a host, which comprises administering intranasally to the host an effective amount of interleukin-12 and an antigen of the pathogen.

22. The method of Claim 21 wherein the pathogen is selected from the group consisting of: bacteria, viruses, mycobacteria, parasites and fungi.

23. The method of Claim 22 wherein the bacteria is selected from the group consisting of: *S. pneumoniae*, *N. meningiditis* and *H. influenza*.

24. The method of Claim 22 wherein the virus is selected from the group consisting of: influenza virus, parainfluenza virus, poliovirus and human immunodeficiency virus.

25. The method of Claim 24 wherein the parasite is selected from the group consisting of: Leishmania, Schistosomes, Trapanosomes, toxoplasma and pneumocystis.

26. The method of Claim 24 wherein the antigen is derived from a toxin of the pathogen.

27. The method of Claim 21 wherein the immune response results in enhanced expression of IgG2a, IgG2b and IgG3 antibodies which are specific to the antigen.

28. A method of inducing a Th1-like immune response to a pathogen in a host, comprising administering intranasally to the host an effective amount of interleukin-12 and an antigen of the pathogen.

29. The method of Claim 28 wherein the pathogen is selected from the group consisting of: bacteria, viruses, mycobacteria, parasites and fungi.

30. The method of Claim 29 wherein the bacteria is selected from the group consisting of: *S. pneumoniae*, *N. meningiditis* and *H. influenza*.

31. The method of Claim 29 wherein the virus is selected from the group consisting of: influenza virus, parainfluenza virus, poliovirus and human immunodeficiency virus.

32. The method of Claim 29 wherein the parasite is selected from the group consisting of: Leishmania, Schistosomes, Trapanosomes, toxoplasma and pneumocystis.

33. The method of Claim 28 wherein the antigen is derived from a toxin of the pathogen.

34. The method of Claim 28 wherein the Th1-like response results in enhanced expression of interferon-γ.

35. The method of Claim 34 wherein the Th1-like immune response results in enhanced expression of IgG2a, IgG2b and IgG3 antibodies which are specific to the antigen.

36. A method of enhancing a mucosal immune response to a pathogen in a host, which comprises administering intranasally to the host an effective amount of interleukin-12 and an antigen of the pathogen.

37. The method of Claim 36 wherein the pathogen is selected from the group consisting of: bacteria, viruses, mycobacteria, parasites and fungi.

38. The method of Claim 37 wherein the bacteria is selected from the group consisting of: *S. pneumoniae*, *N. meningiditis* and *H. influenza*.

39. The method of Claim 37 wherein the virus is selected from the group consisting of: influenza virus, parainfluenza virus, poliovirus and human immunodeficiency virus.

40. The method of Claim 37 wherein the parasite is selected from the group consisting of: Leishmania, Schistosomes, Trapanosomes, toxoplasma and pneumocystis.

41. The method of Claim 36 wherein the antigen is derived from a toxin of the pathogen.

42. The method of Claim 36 wherein the immune response is a Th1-type cytokine response.

43. The method of Claim 42 wherein the Th1-type cytokine response results in enhanced expression of interferon-γ in the host.

44. The method of Claim 36 wherein the immune response is a humoral immune response.

45. The method of Claim 44 wherein the humoral immune response results in enhanced expression of IgG2a, IgG2b and IgG3 antibodies which are specific to the antigen.

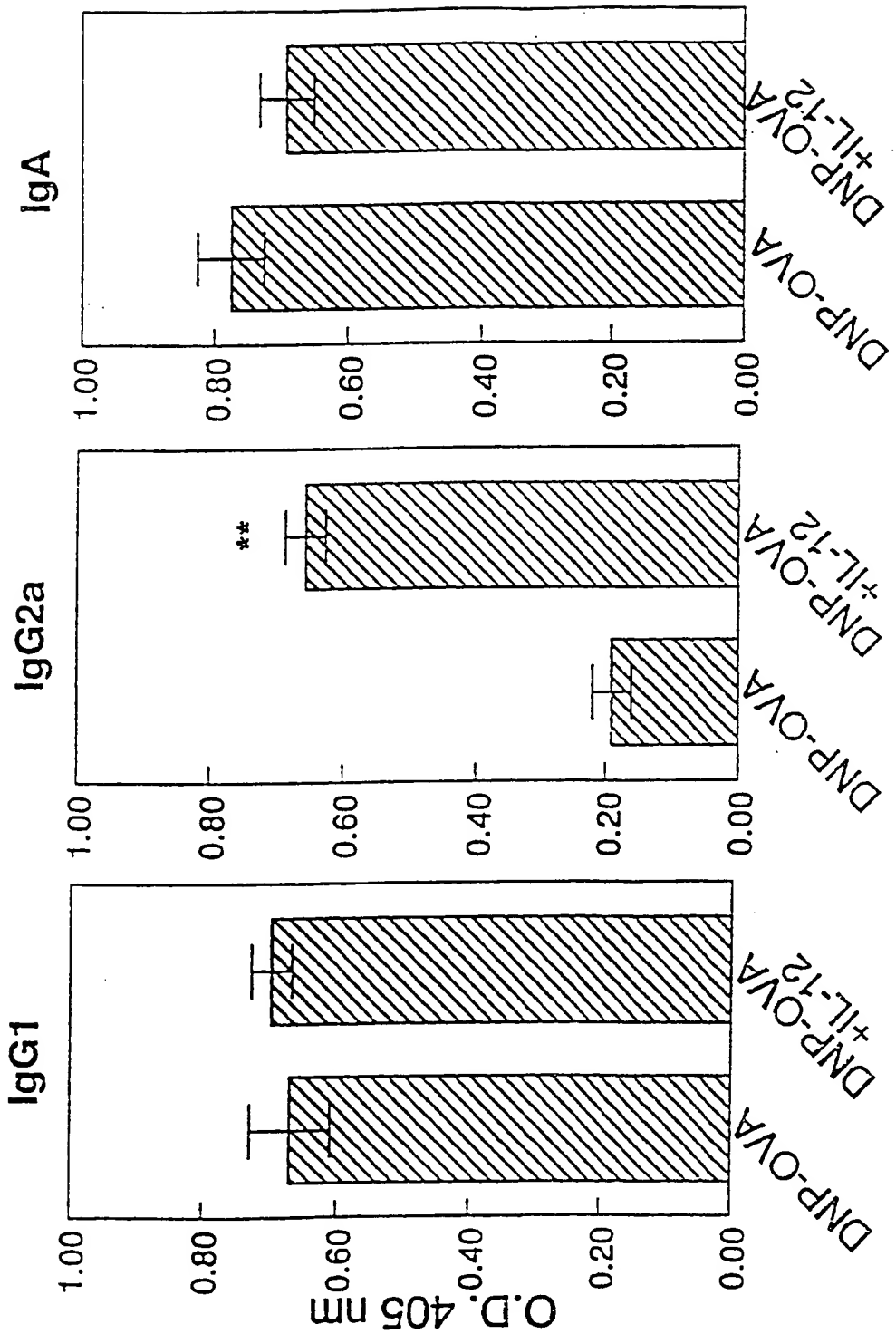
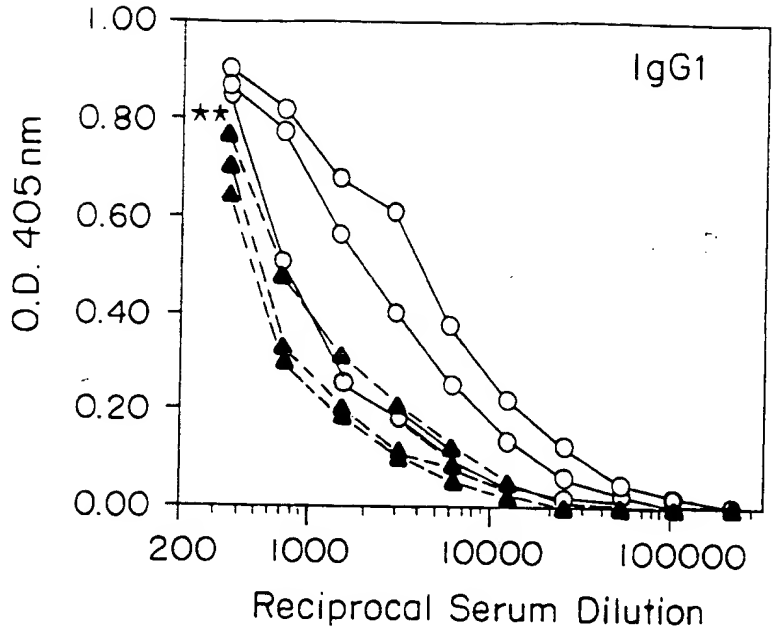


Fig. 1A

Fig. 1B

Fig. 1C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0.80
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IgG1
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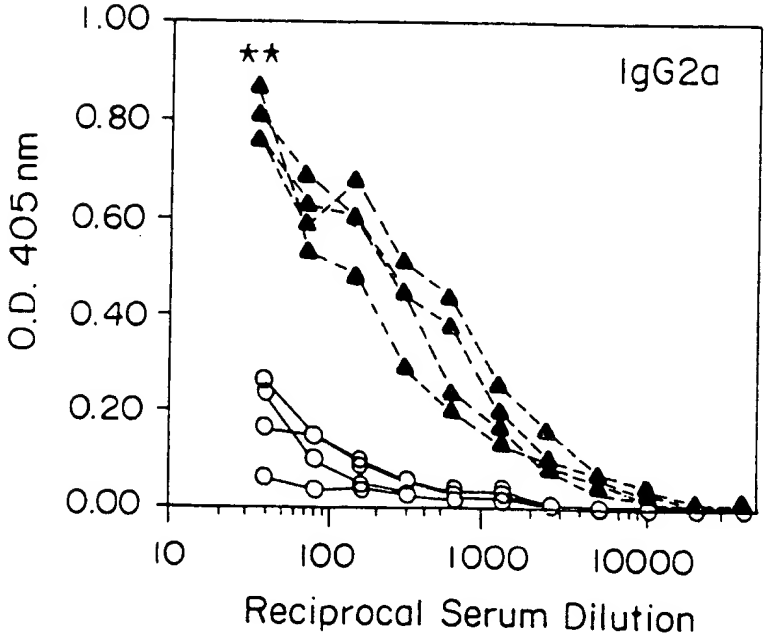
1000
10000
100000
Reciprocal Serum Dilution

FIG. 2A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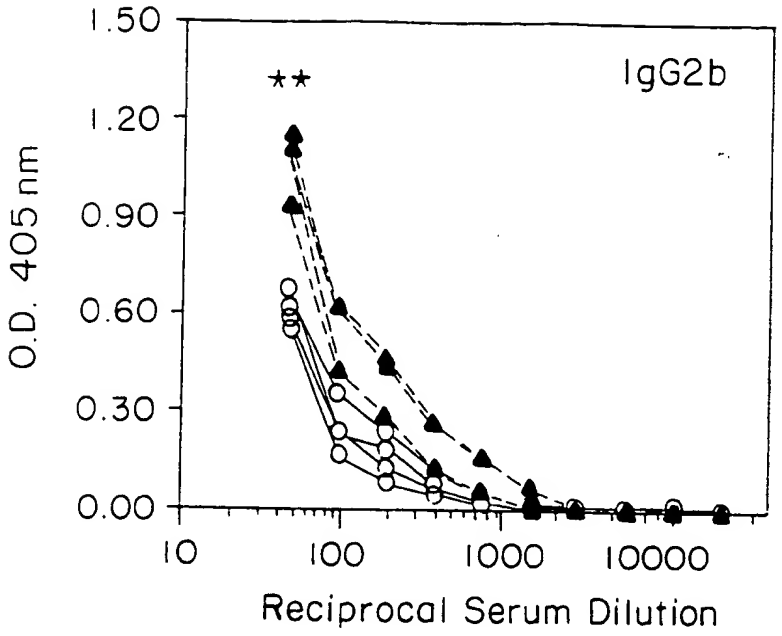
Reciprocal Serum Dilution

FIG. 2B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1.20
0.90
0.60
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0.00
O.D. 405 nm
**
IgG2b
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100
1000
10000
Reciprocal Serum Dilution

FIG. 2C

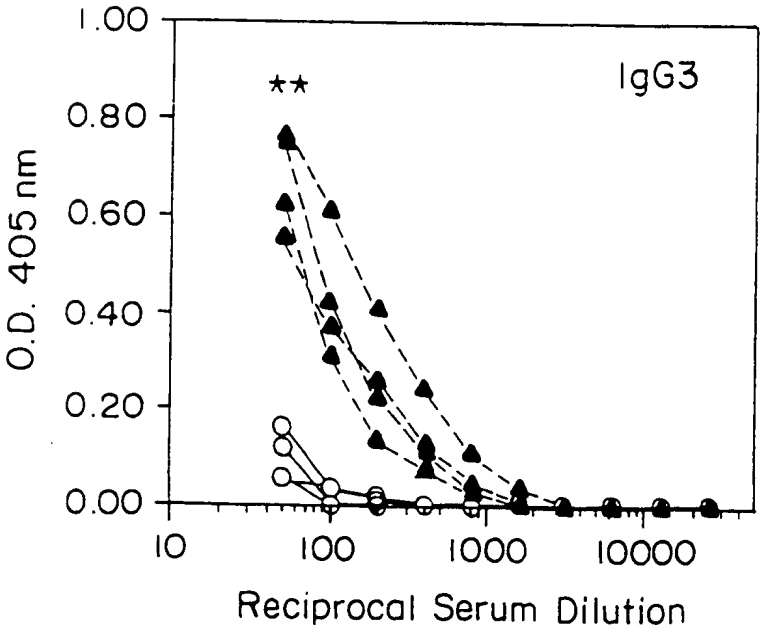

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
**
IgG3
10
100
1000
10000
Reciprocal Serum Dilution

FIG. 2D

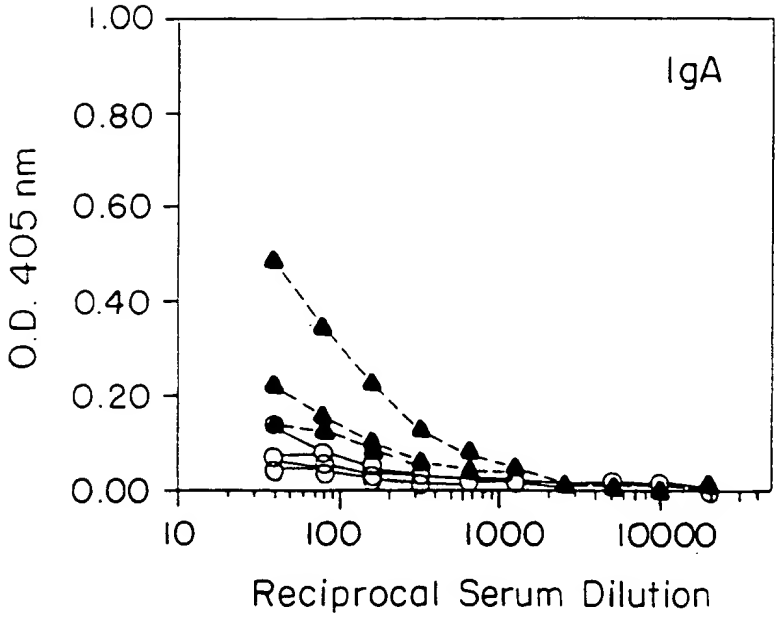

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
IgA
10
100
1000
10000
Reciprocal Serum Dilution

FIG. 2E

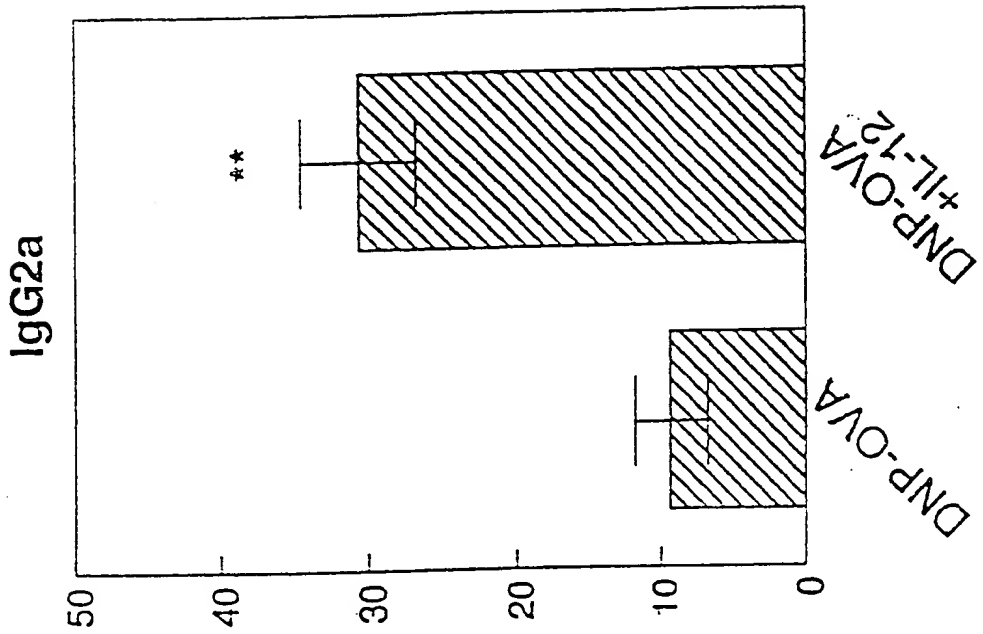

IgG2a
50
40
30
20
10
0
**
DNP-OVA
DNP-OVA +IL-12

Fig. 3B

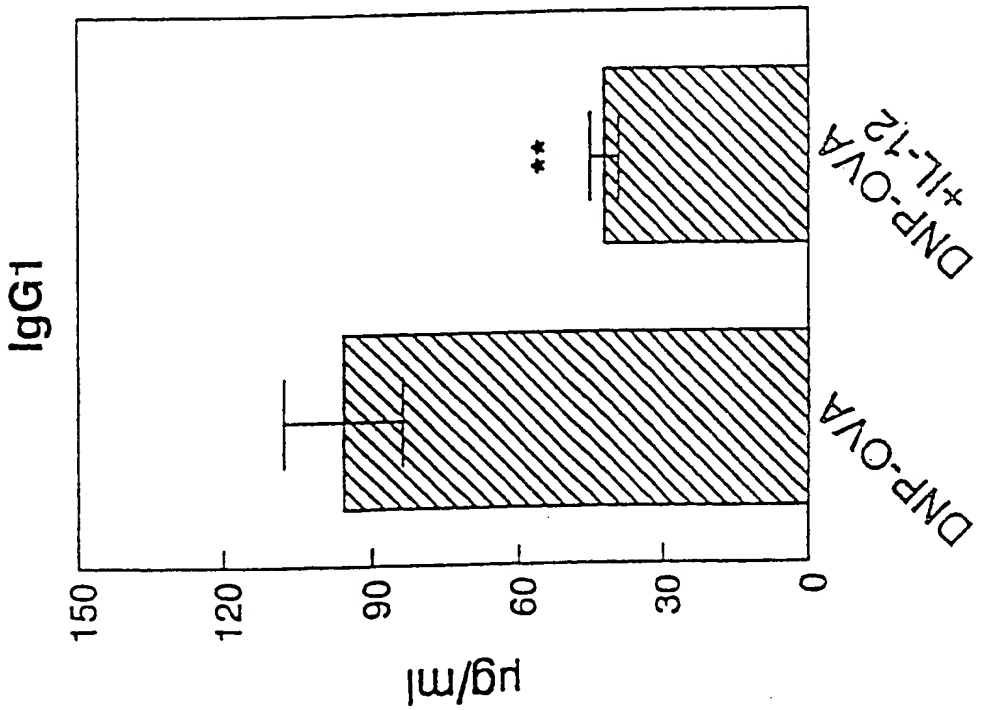

IgG1
150
120
90
60
30
0
μg/ml
**
DNP-OVA
DNP-OVA +IL-12

Fig. 3A

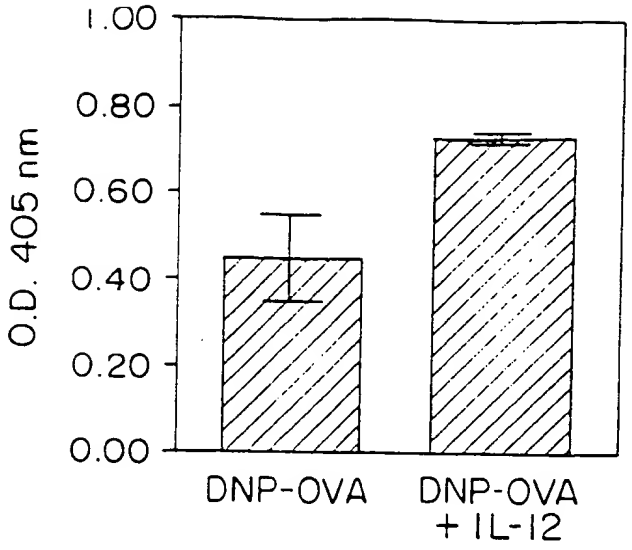

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
DNP-OVA
DNP-OVA
+ IL-12

FIG. 4A

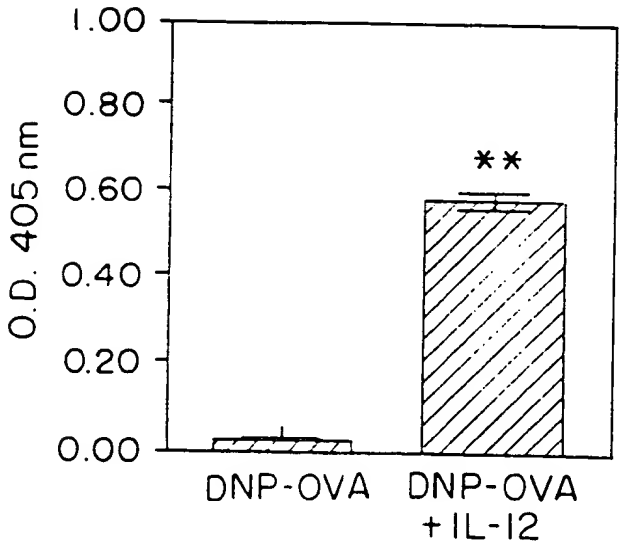

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
**
DNP-OVA
DNP-OVA
+ IL-12

FIG. 4B

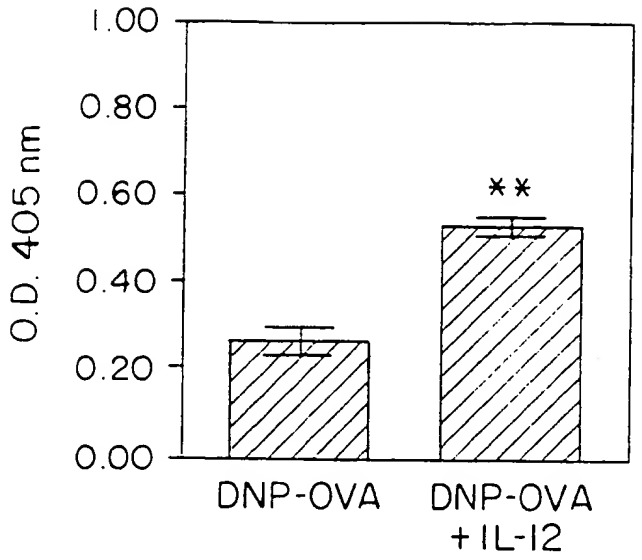

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
**
DNP-OVA
DNP-OVA
+IL-12

FIG. 4C

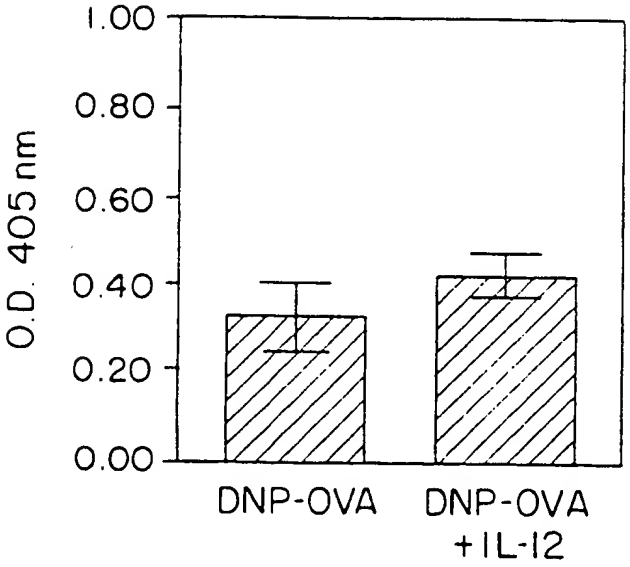

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
DNP-OVA
DNP-OVA
+IL-12

FIG. 4D

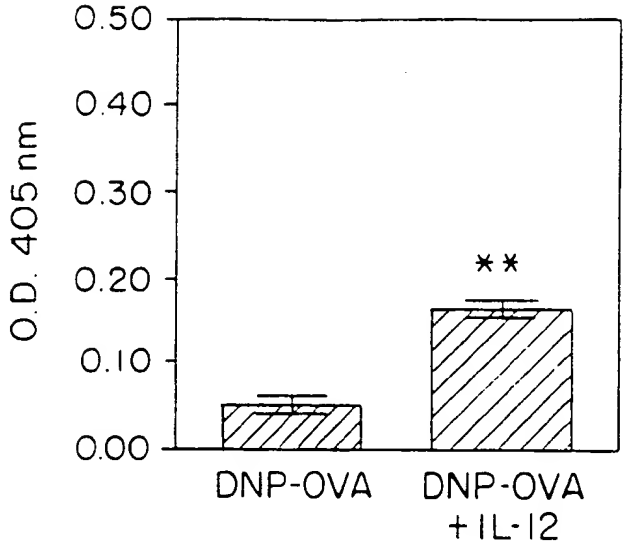

0.50
0.40
0.30
0.20
0.10
0.00
O.D. 405 nm
**
DNP-OVA
DNP-OVA
+IL-12

FIG. 4E

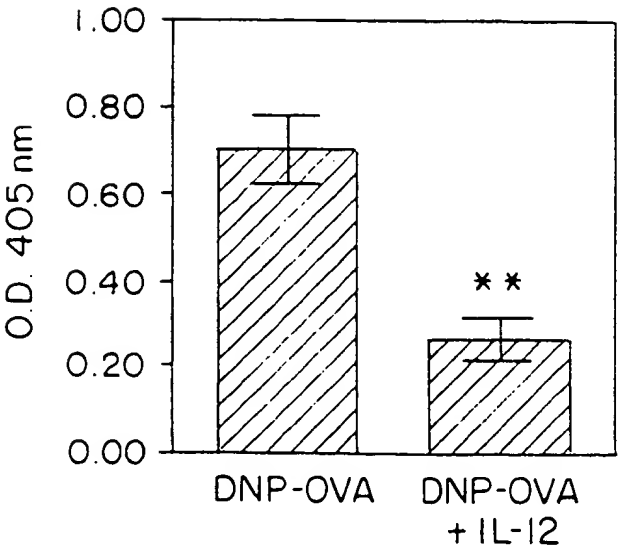

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
**
DNP-OVA
DNP-OVA
+IL-12

FIG. 4F

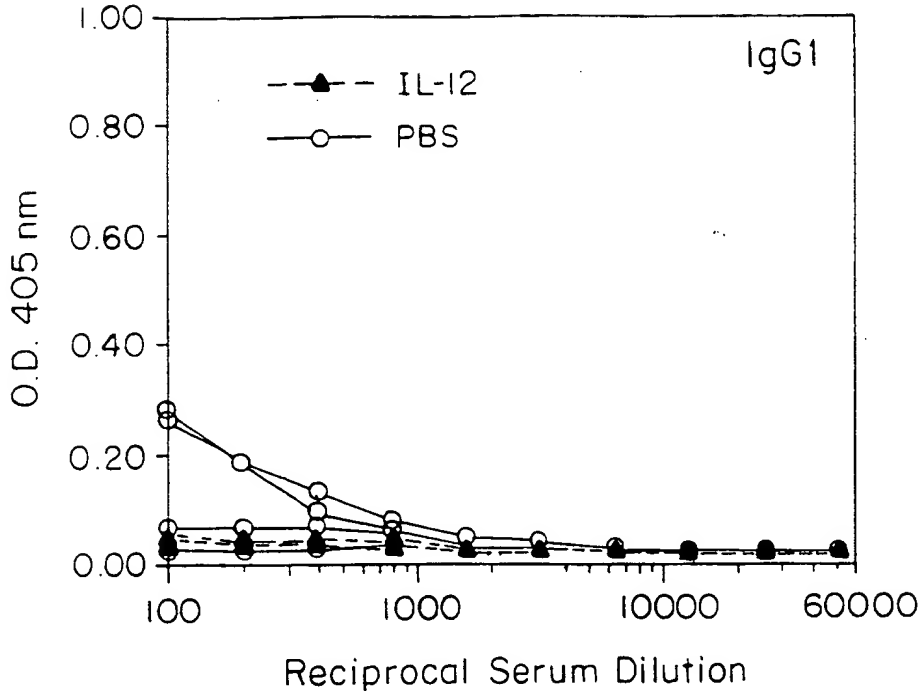

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
IgG1
IL-12
PBS
100
1000
10000
60000
Reciprocal Serum Dilution

FIG. 5A

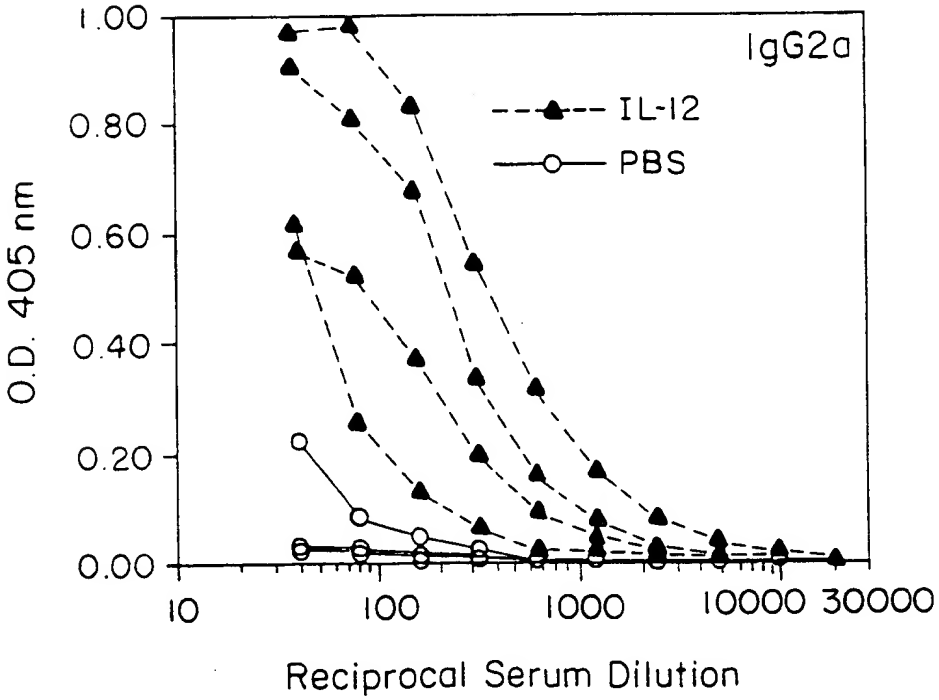

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
IgG2a
IL-12
PBS
10
100
1000
10000 30000
Reciprocal Serum Dilution

FIG. 5B

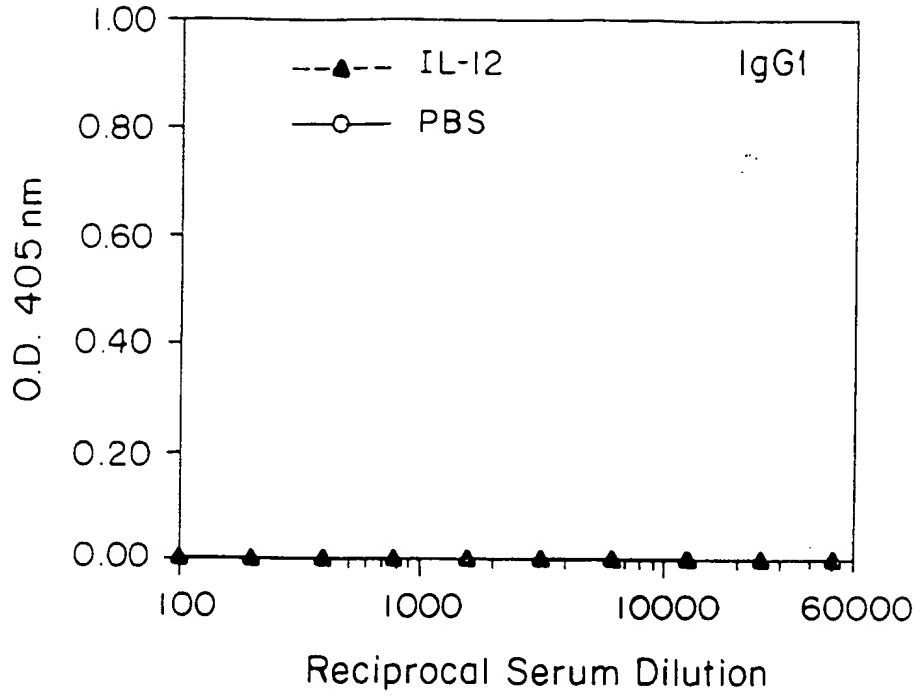

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
IL-12
PBS
IgG1
100
1000
10000
60000
Reciprocal Serum Dilution

FIG. 6A

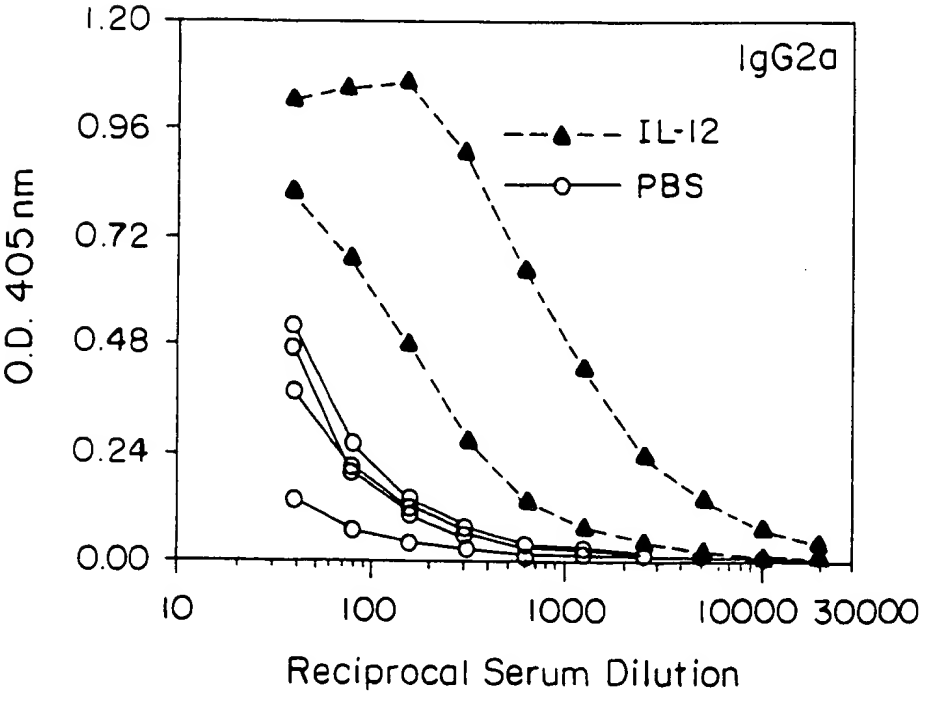

1.20
0.96
0.72
0.48
0.24
0.00
O.D. 405 nm
IgG2a
IL-12
PBS
10
100
1000
10000
30000
Reciprocal Serum Dilution

FIG. 6B

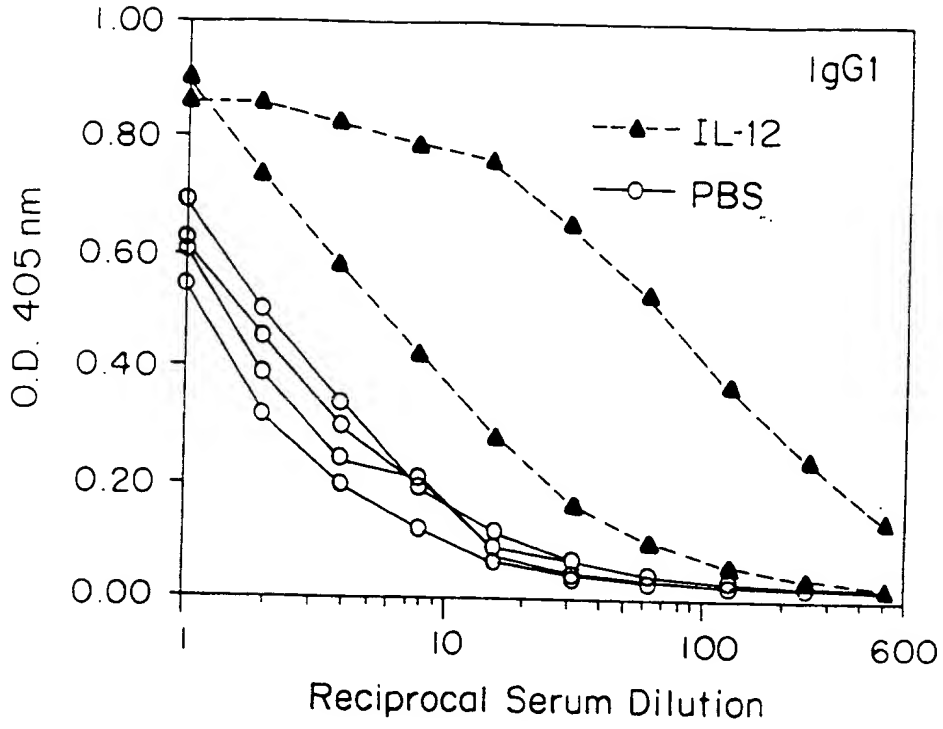

1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
IgG1
IL-12
PBS
1
10
100
600
Reciprocal Serum Dilution

FIG. 7A

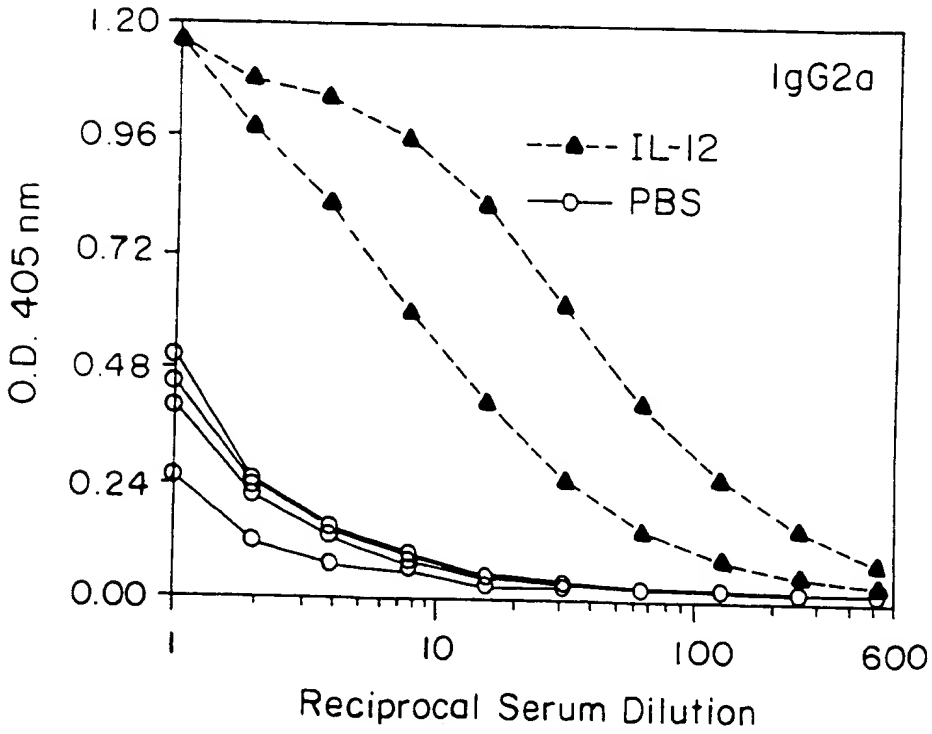

1.20
0.96
0.72
0.48
0.24
0.00
O.D. 405 nm
IgG2a
IL-12
PBS
1
10
100
600
Reciprocal Serum Dilution

FIG. 7B

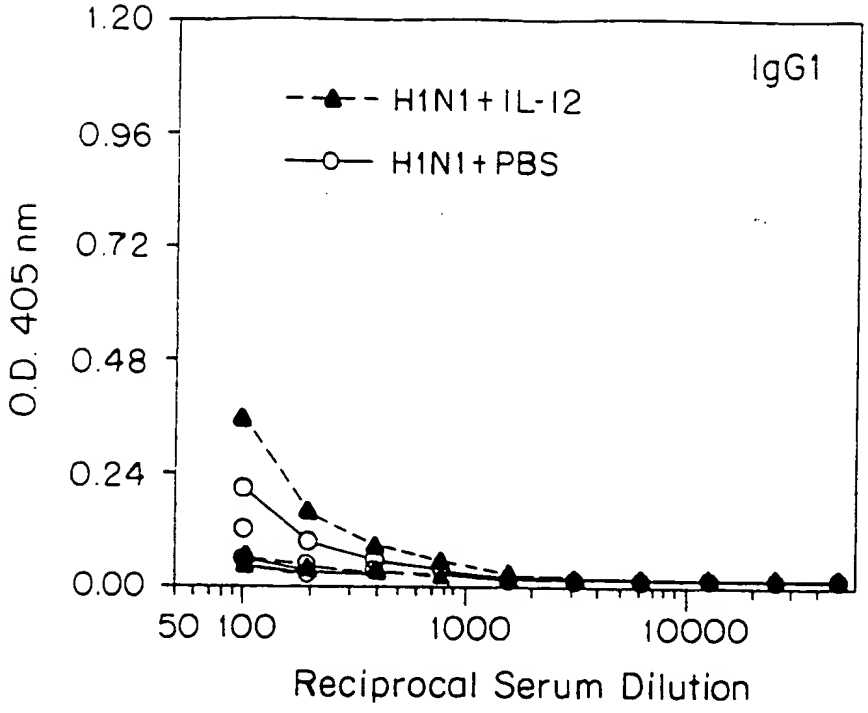

1.20
0.96
0.72
0.48
0.24
0.00
O.D. 405 nm
IgG1
H1N1+IL-12
H1N1+PBS
50 100
1000
10000
Reciprocal Serum Dilution

FIG. 8A

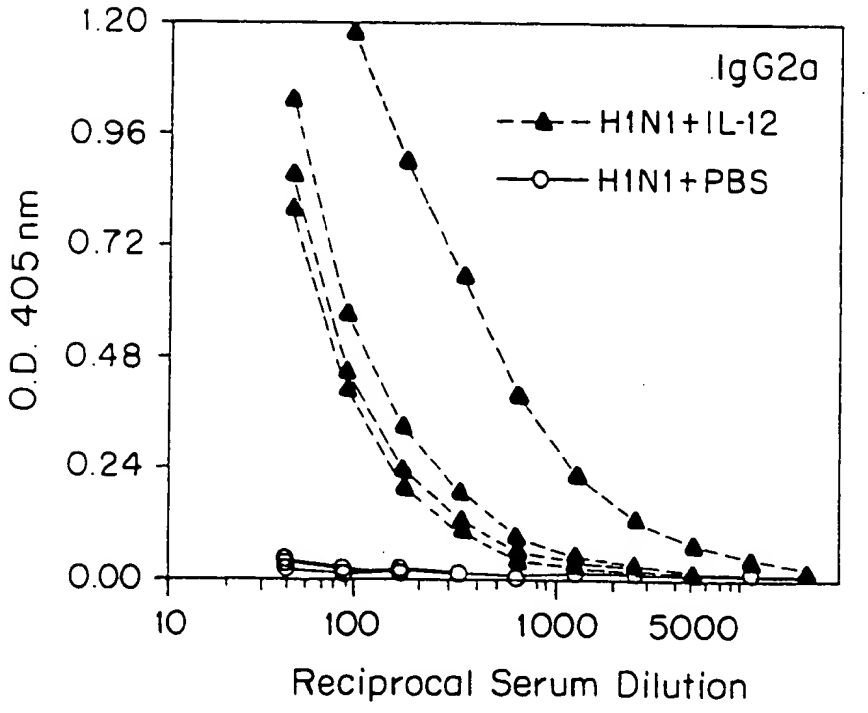

1.20
0.96
0.72
0.48
0.24
0.00
O.D. 405 nm
IgG2a
H1N1+IL-12
H1N1+PBS
10
100
1000
5000
Reciprocal Serum Dilution

FIG. 8B

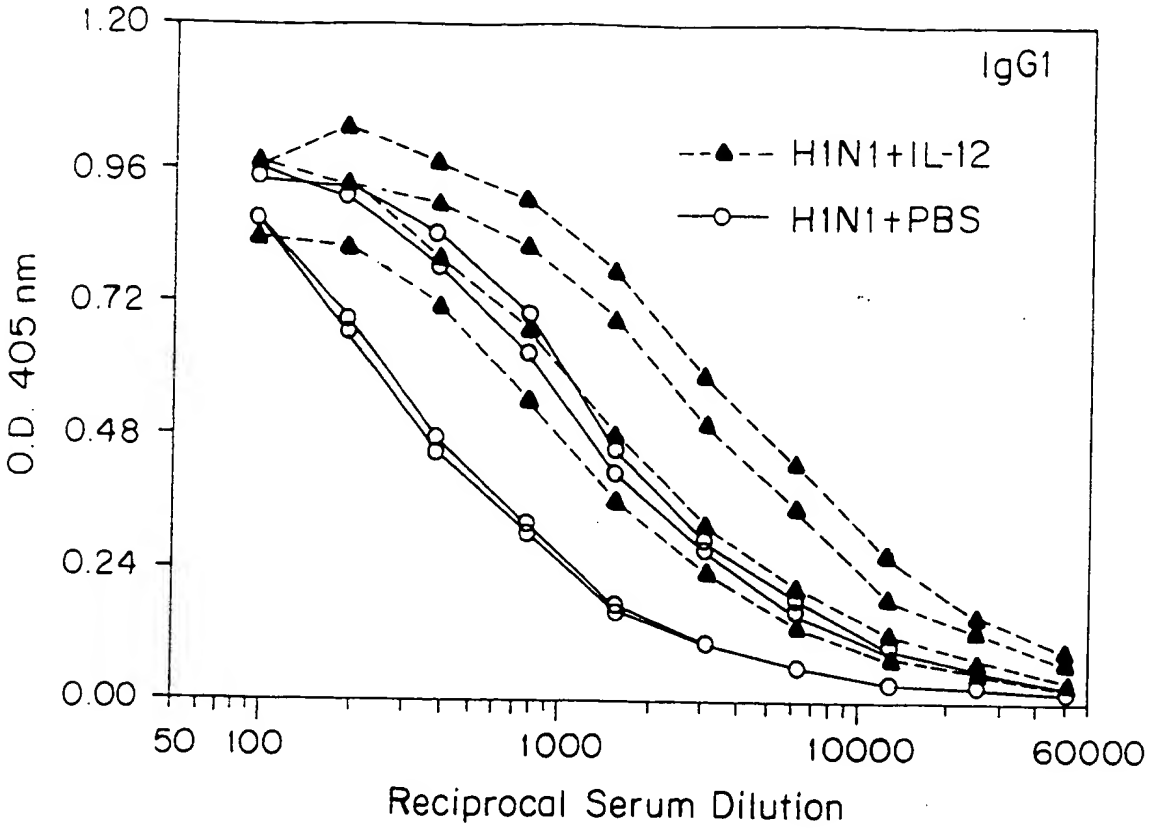

IgG1
H1N1+IL-12
H1N1+PBS
O.D. 405 nm
1.20
0.96
0.72
0.48
0.24
0.00
50
100
1000
10000
60000
Reciprocal Serum Dilution

FIG. 9A

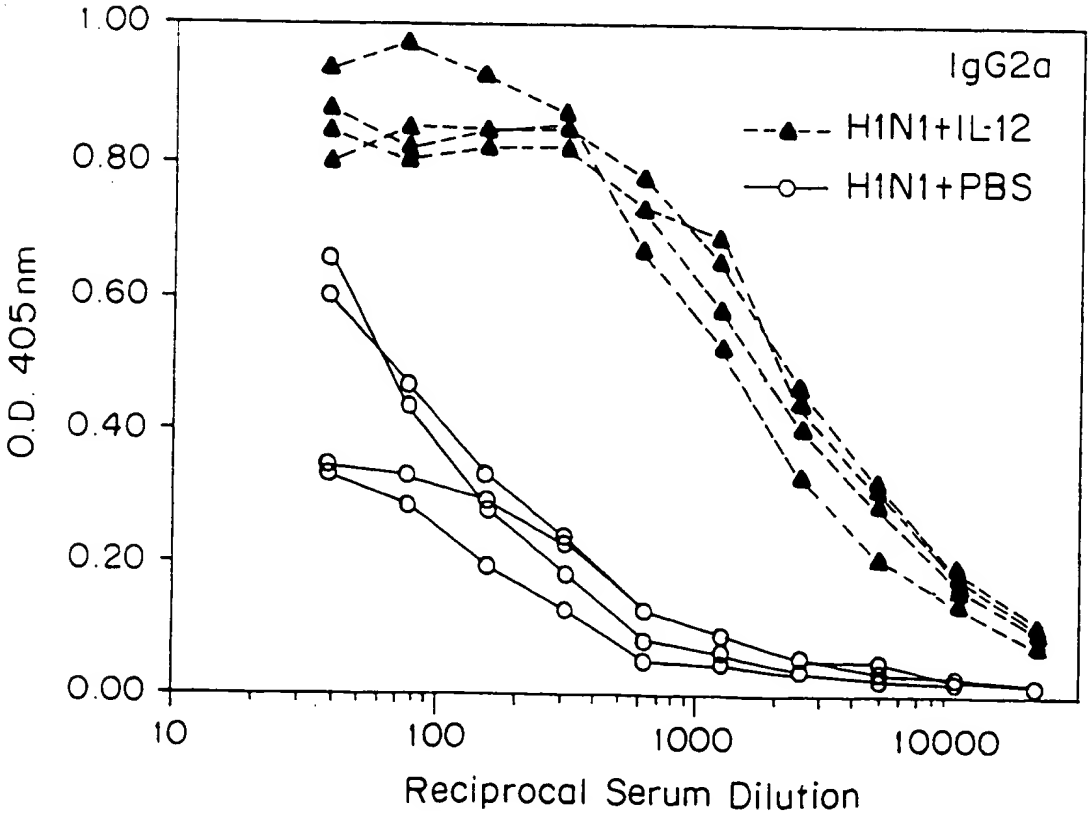

IgG2a
H1N1+IL-12
H1N1+PBS
O.D. 405nm
1.00
0.80
0.60
0.40
0.20
0.00
10
100
1000
10000
Reciprocal Serum Dilution

FIG. 9B

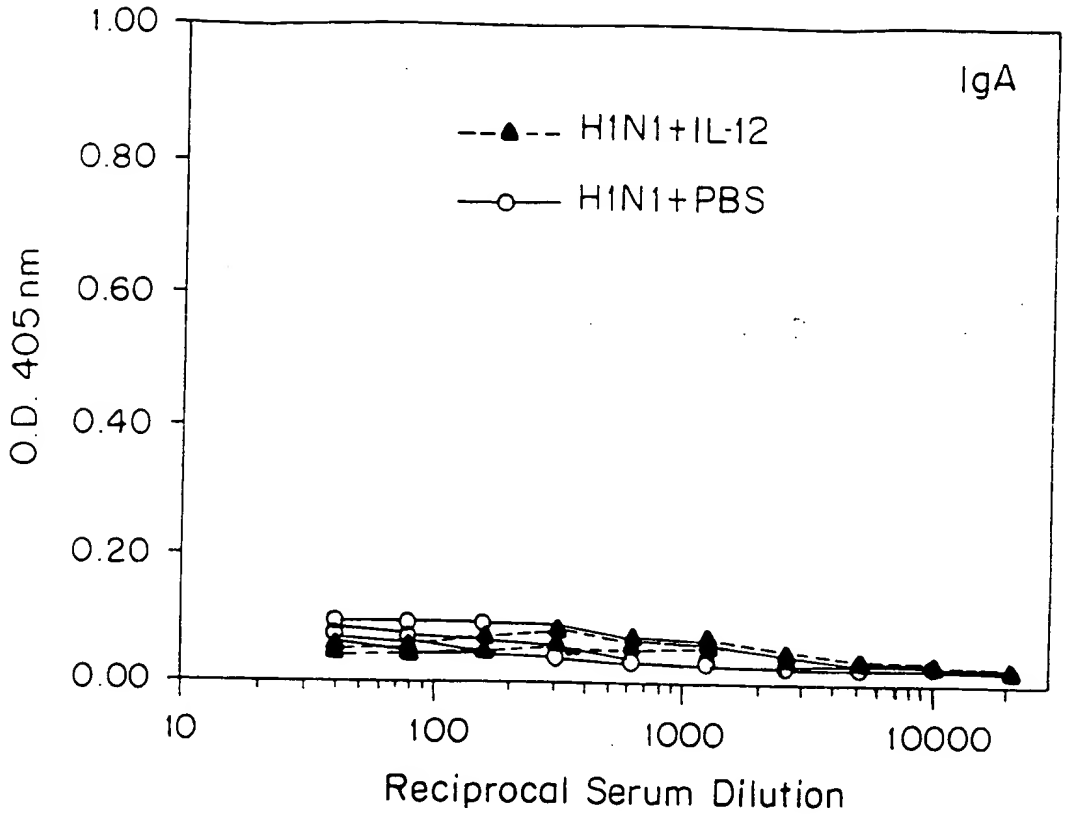
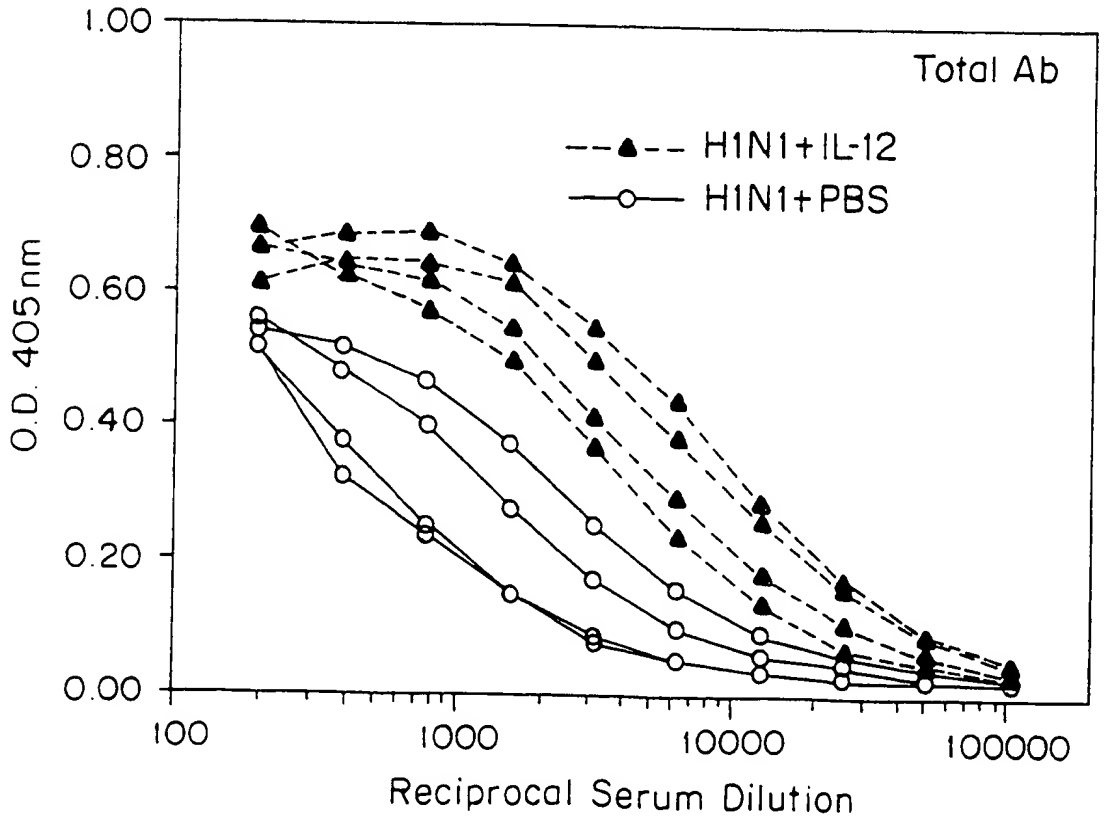

IgA
H1N1+IL-12
H1N1+PBS
O.D. 405 nm
1.00
0.80
0.60
0.40
0.20
0.00
10
100
1000
10000
Reciprocal Serum Dilution

FIG. 9C


Total Ab
H1N1+IL-12
H1N1+PBS
O.D. 405nm
1.00
0.80
0.60
0.40
0.20
0.00
100
1000
10000
100000
Reciprocal Serum Dilution

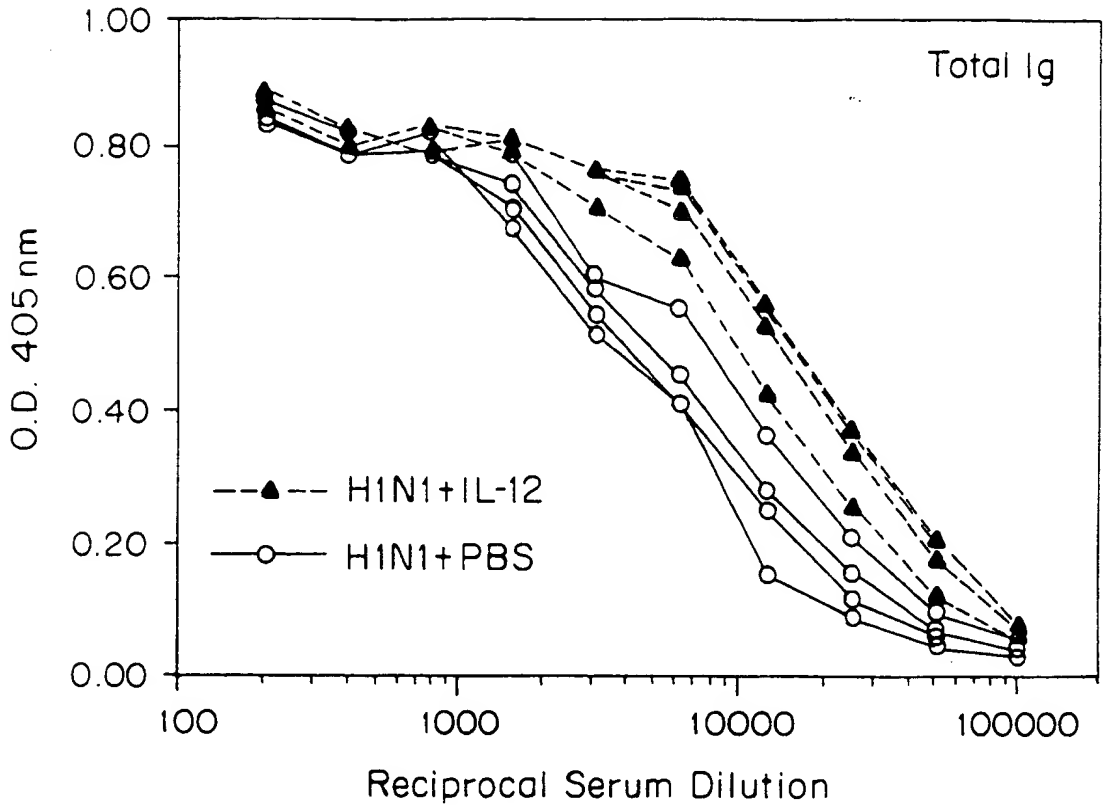

Total Ig
1.00
0.80
0.60
0.40
0.20
0.00
O.D. 405 nm
H1N1+IL-12
H1N1+PBS
100
1000
10000
100000
Reciprocal Serum Dilution

FIG. 9E

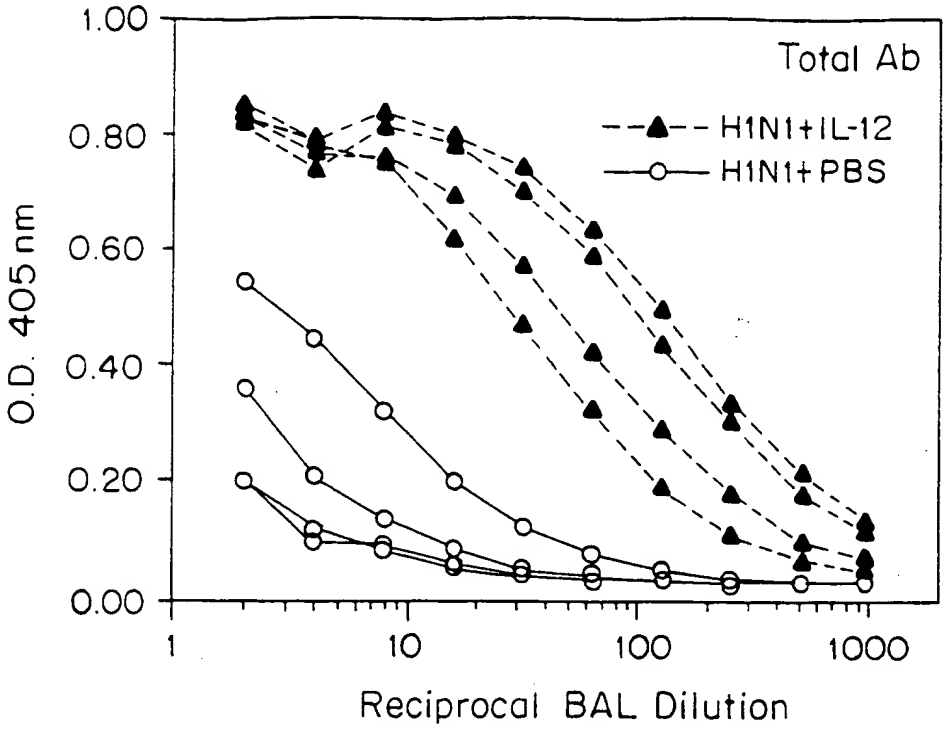


FIG. 10A

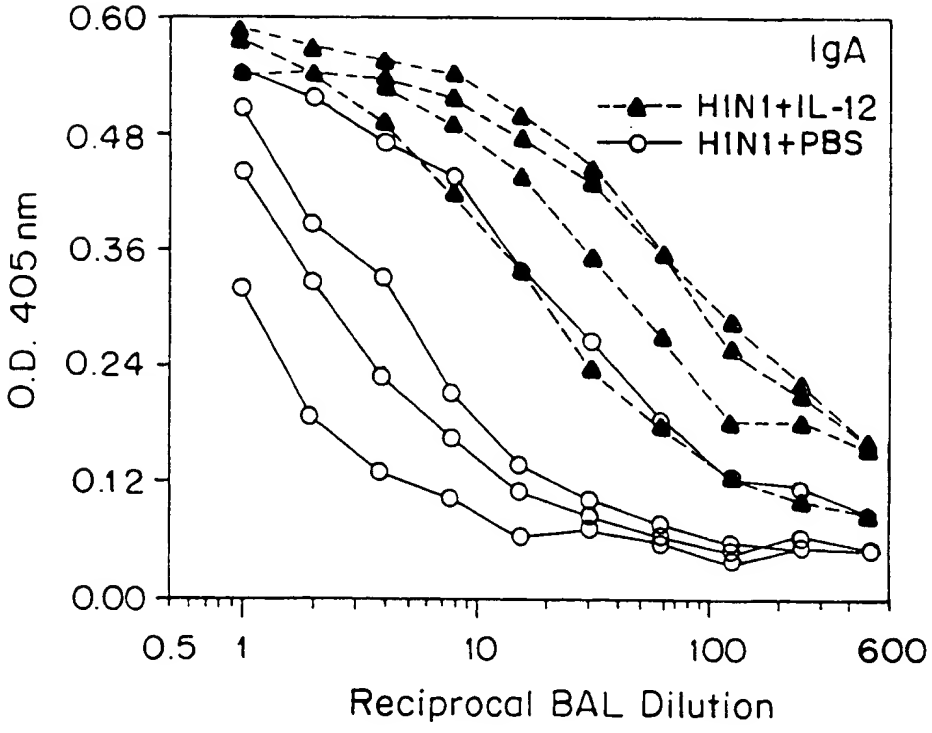


FIG. 10B

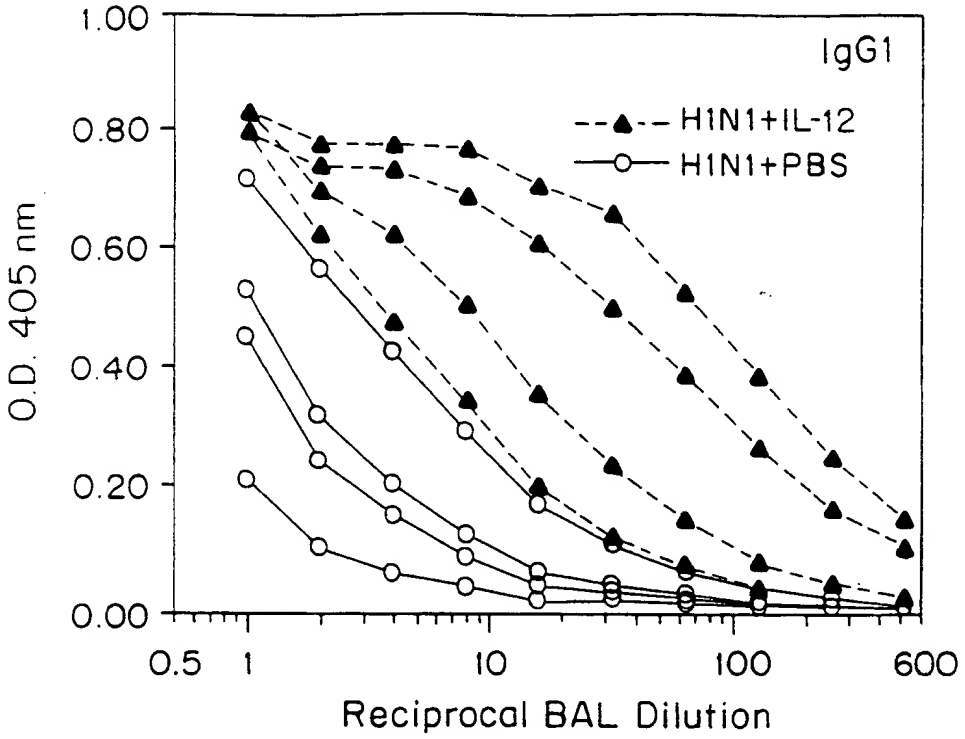


FIG. 10C

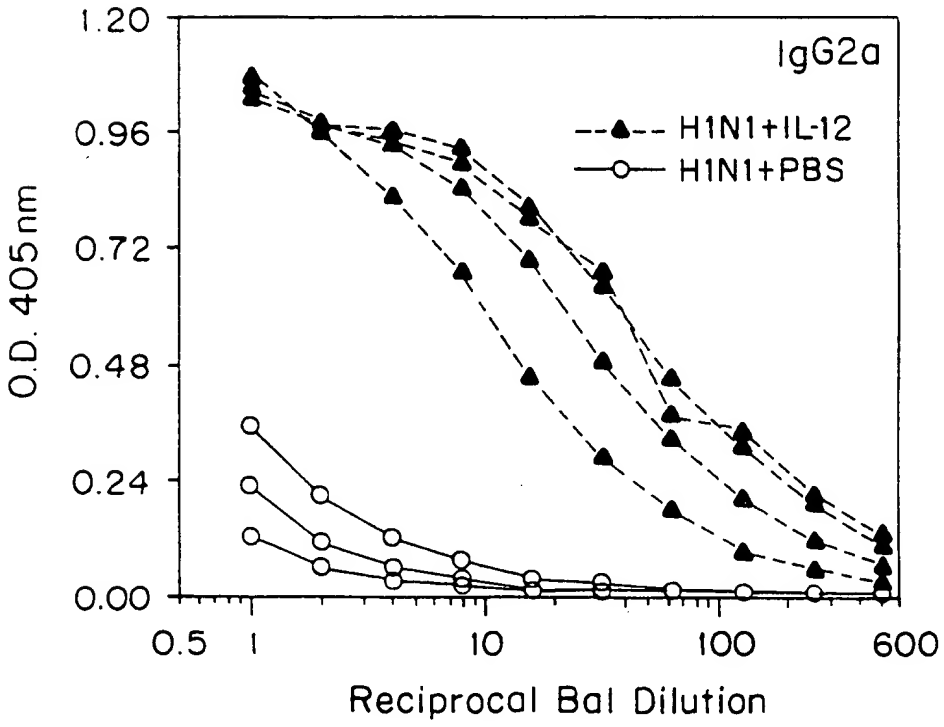


FIG. 10D

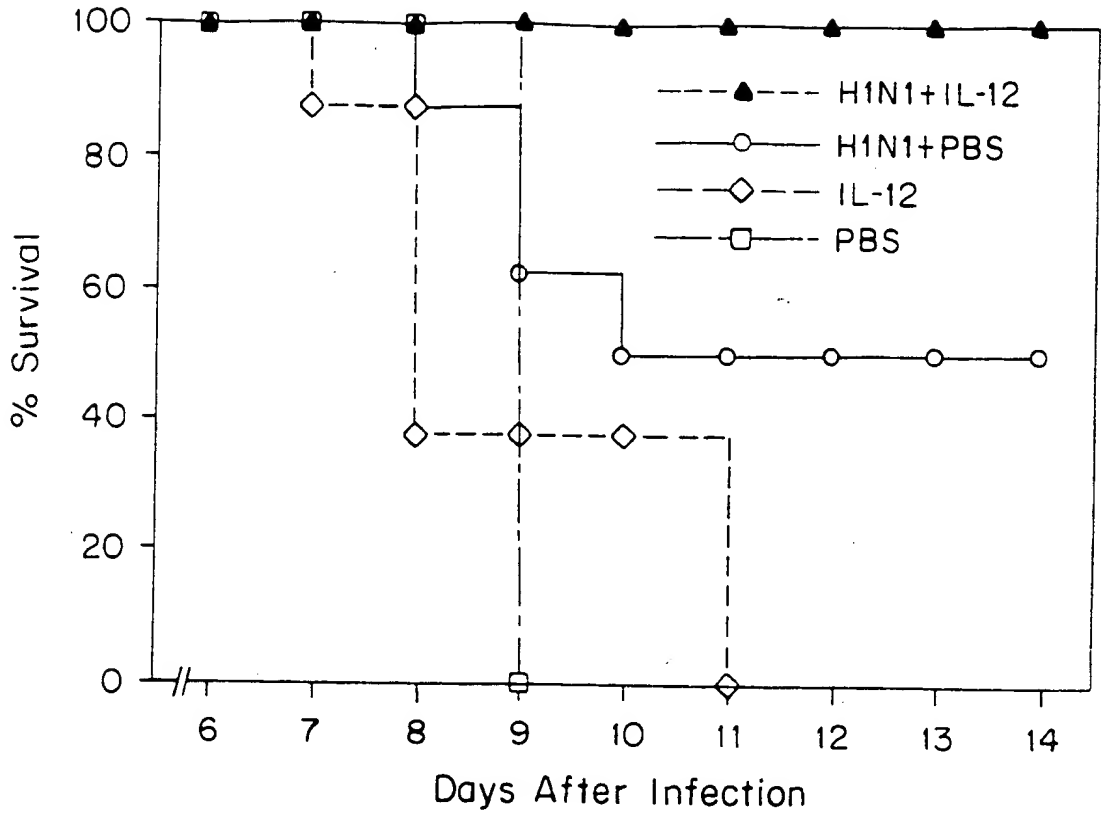


FIG. 11A

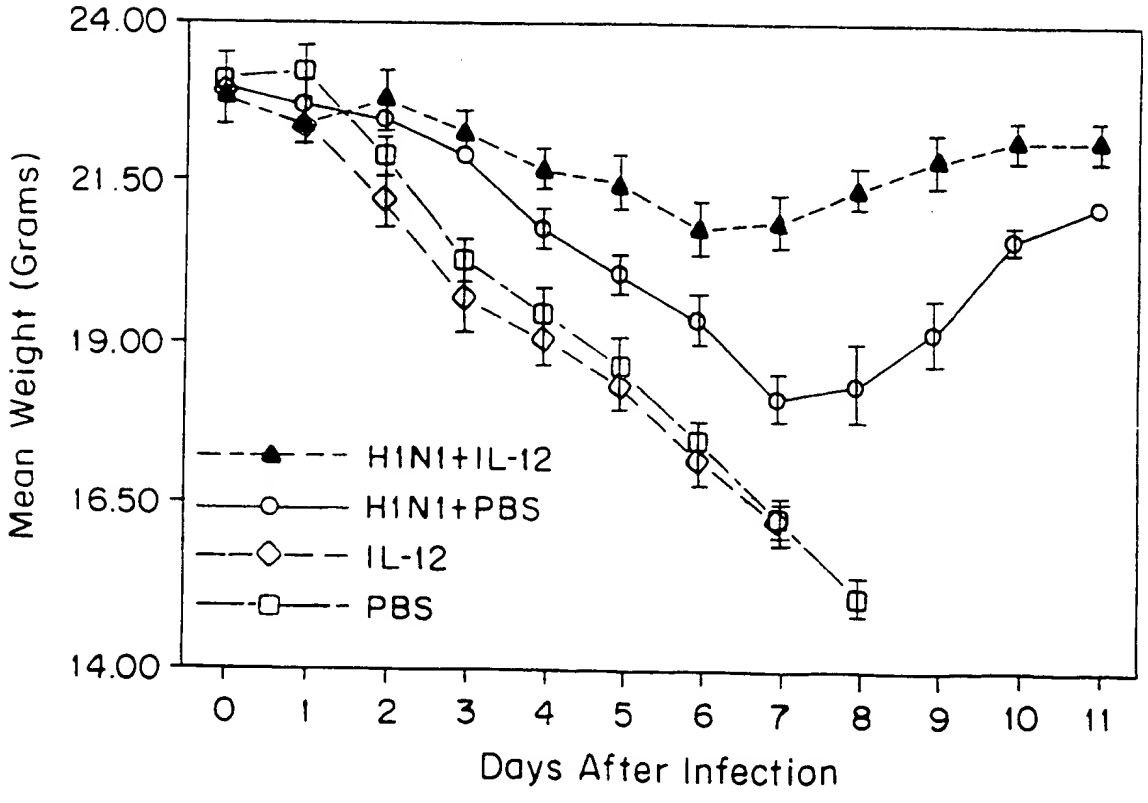


FIG. 11B

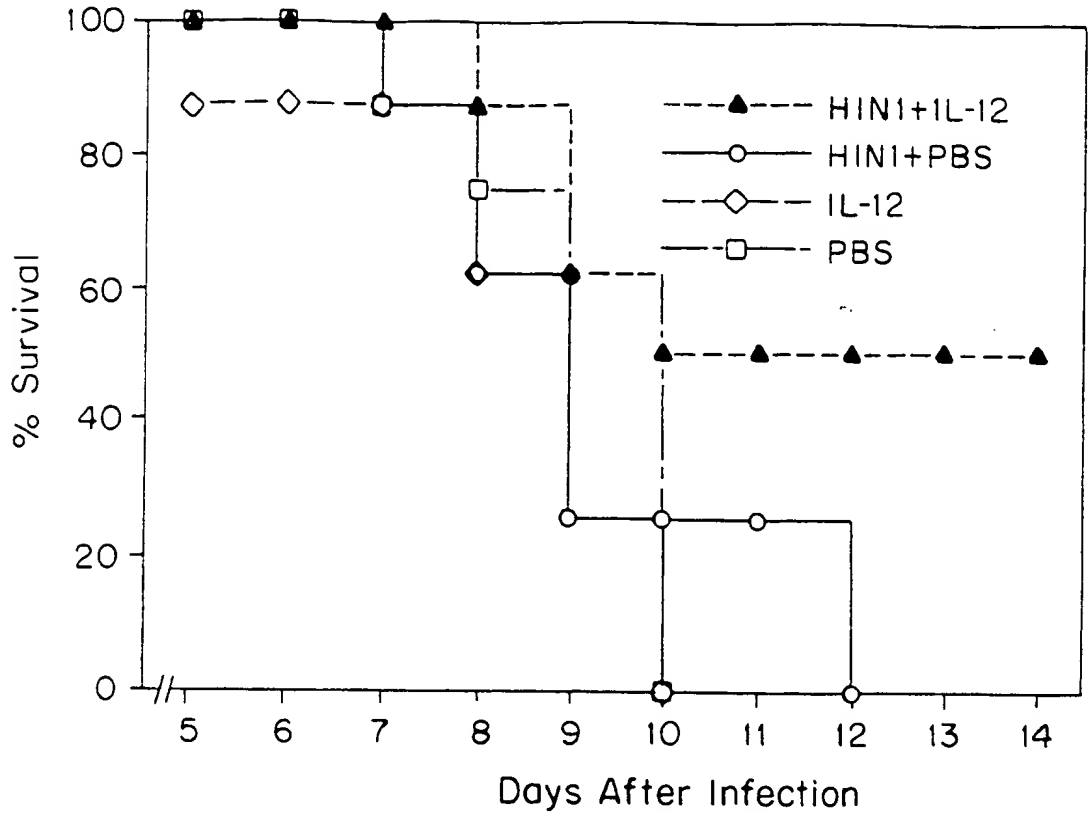


FIG. 11C

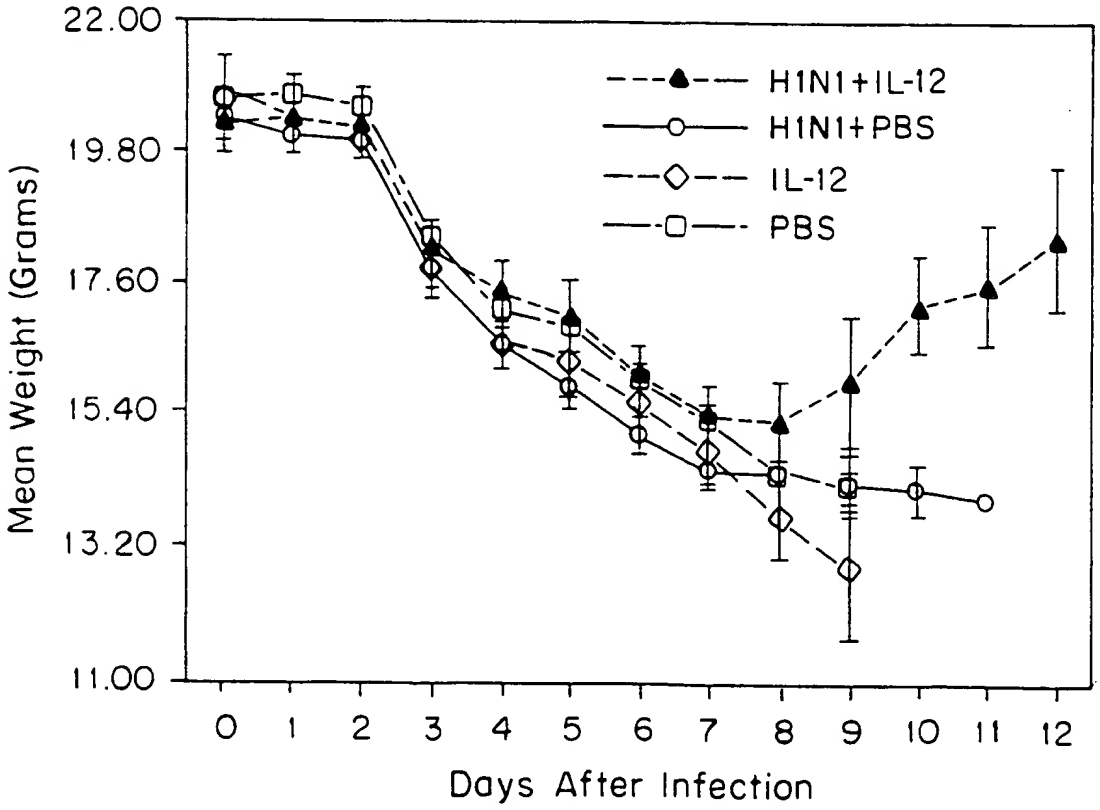


FIG. 11D

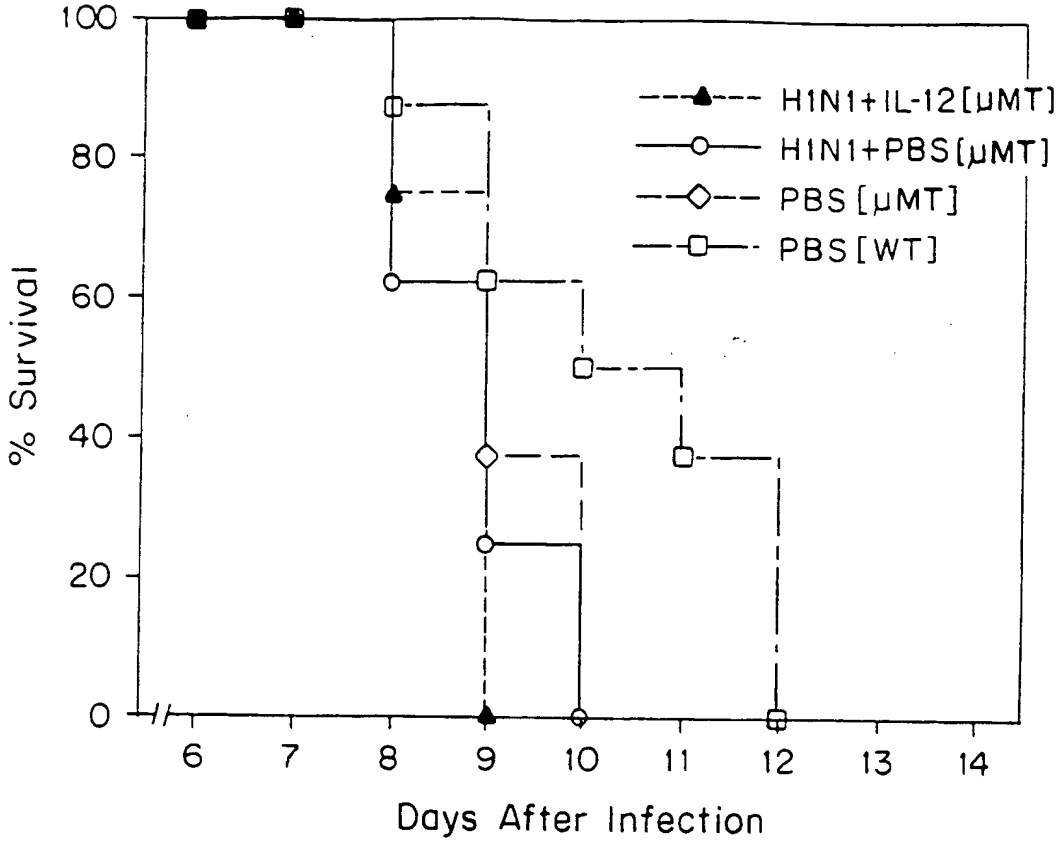


FIG. 12A

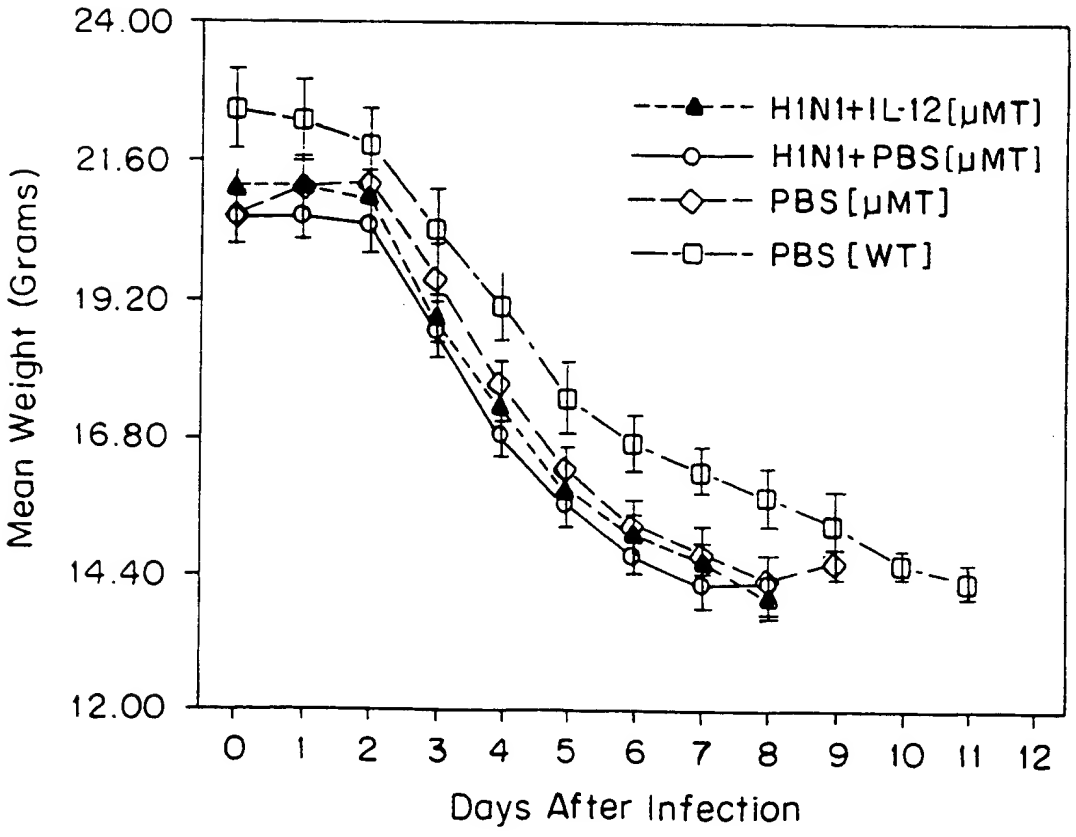


FIG. 12B

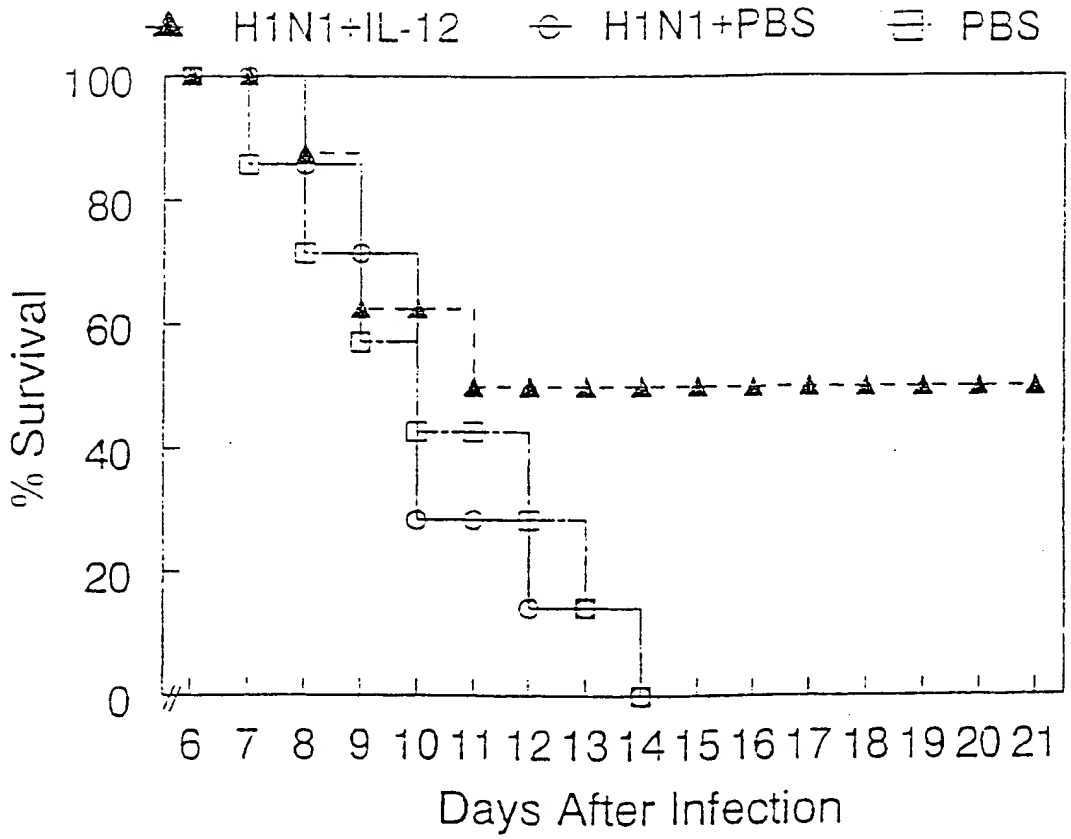

H1N1+IL-12
H1N1+PBS
PBS
100
80
60
40
20
0
% Survival
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days After Infection

Fig. 13

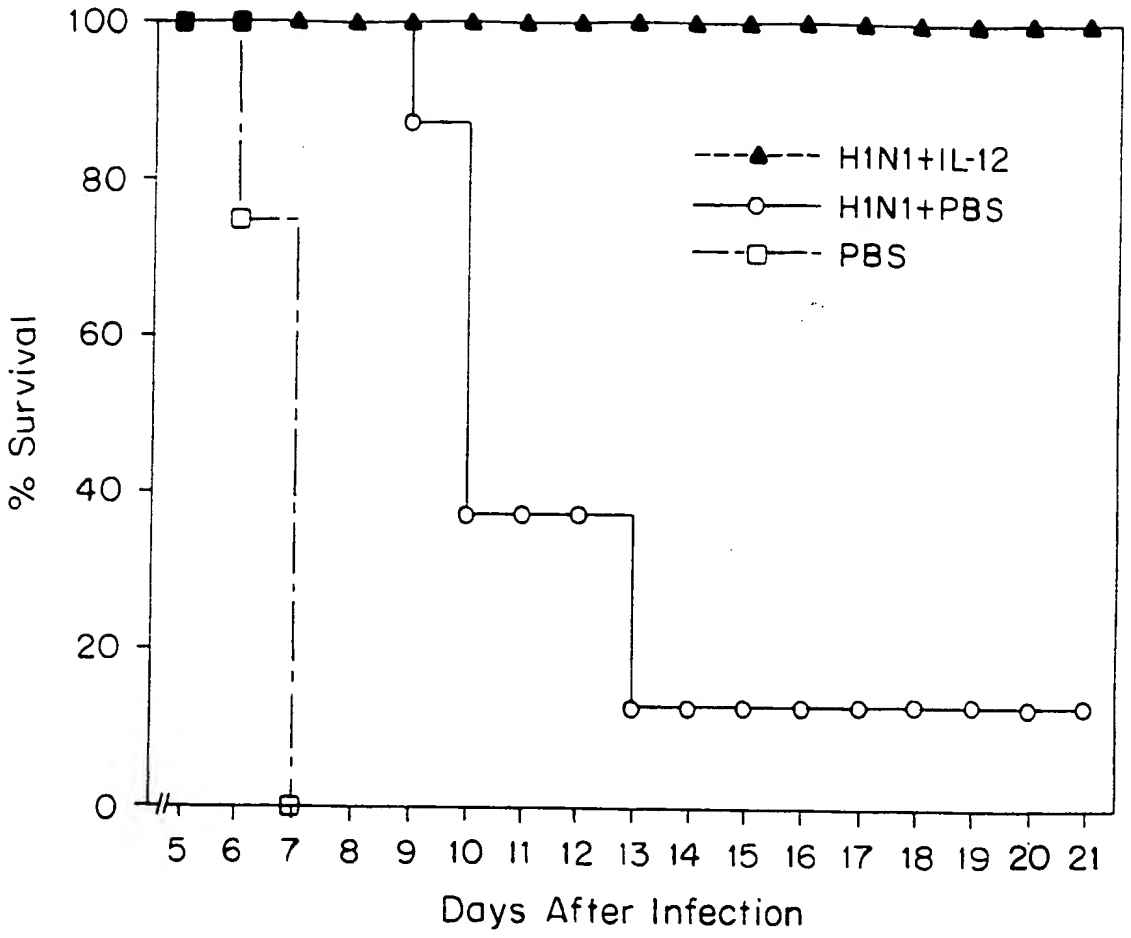

100
80
60
40
20
0
% Survival
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days After Infection
H1N1+IL-12
H1N1+PBS
PBS

FIG. 14A

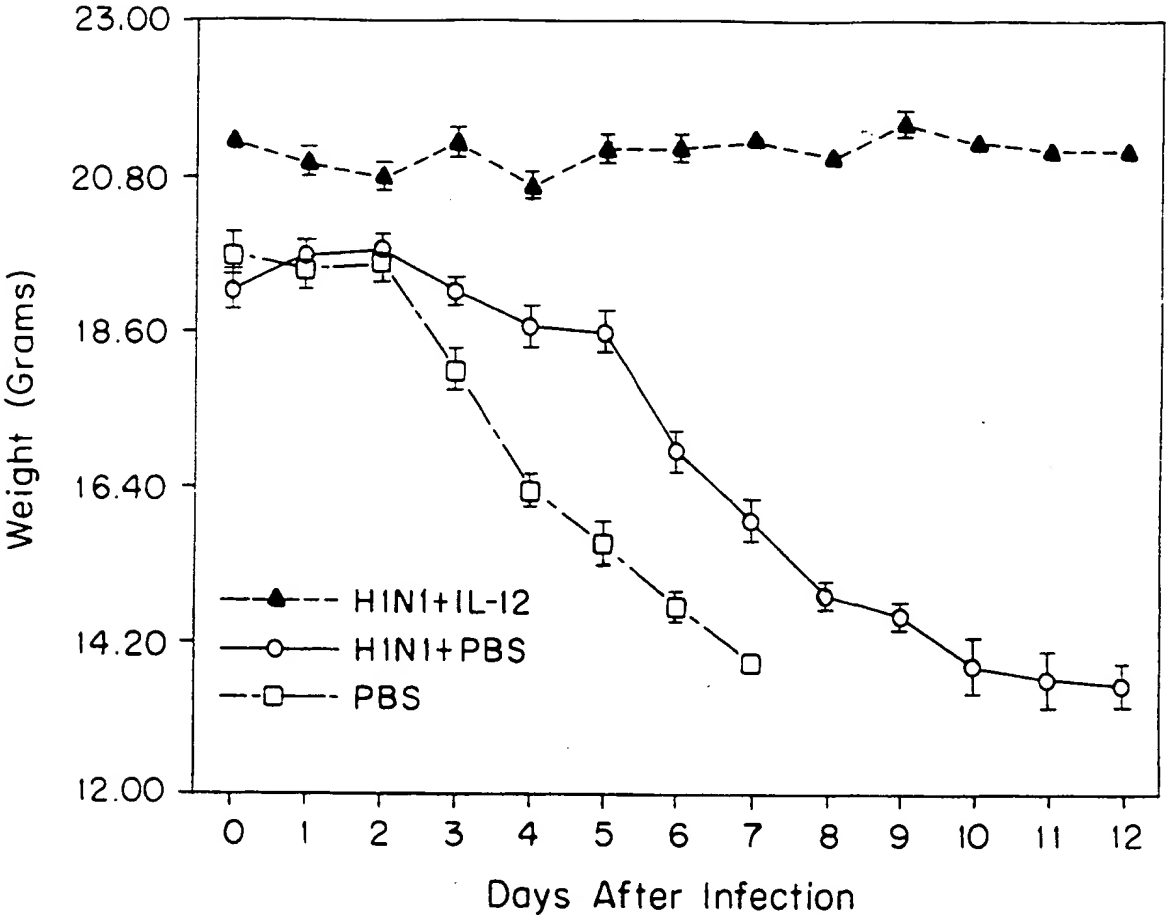

23.00
20.80
18.60
16.40
14.20
12.00
Weight (Grams)
H1N1+IL-12
H1N1+PBS
PBS
0 1 2 3 4 5 6 7 8 9 10 11 12
Days After Infection

FIG. 14B

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 99/04678

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 A61K39/02 A61K39/145 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OKADA E ET AL: "Intranasal immunization of a DNA vaccine with IL - 12 - and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens." JOURNAL OF IMMUNOLOGY, (1997 OCT 1) 159 (7) 3638-47. JOURNAL CODE: IFB. ISSN: 0022-1767., XP002106202 United States see the whole document ---	1-45
X	WO 97 45139 A (GENETICS INST) 4 December 1997 see the whole document, especially page 15 lines 6-19 ---	1-45
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 1999	22/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040 Tx 31 651 epo nl.	Fernandez y Pranas, F

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/US 99/04678

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropnate, of the relevant passages	Relevant to claim No.
X	US 5 723 127 A (SCOTT PHILLIP ET AL) 3 March 1998 see the whole document, especially column 6 lines 34-38	1-45
X,P	ARULANANDAM B.P. ET AL: "Modulation of mucosal and systemic immunity by intranasal interleukin 12 delivery" VACCINE, vol. 17, January 1999, pages 252-260, XP002106203 see the whole document	1-45
A	MARINARO M ET AL: "ORAL BUT NOT PARENTERAL INTERLEUKIN (IL)-12 REDIRECTS T HELPER 2 (TH2)-TYPE RESPONSES TO AN ORAL VACCINE WITHOUT ALTERING MUCOSAL IGA RESPONSES" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 185, no. 3, 3 February 1997, pages 415-427, XP002070004 see abstract see page 418, "Oral IL-12 liposome effects on systemic Ab responses to an oral vaccine" see page 419, column 1, paragraph 1 see page 422, column 2, paragraph 2 see page 424, column 2, paragraph 3	1-45
A	MARINARO M ET AL: "INTRANASAL IL-12 DELIVERY ENHANCES TH2-TYPE WHILE ORAL IL-12 REDIRECTS TH2- TO TH1-TYPE RESPONSES" JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, vol. 99, no. 1, 21 February 1997, page S34 XP002070005 see abstract	1-45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/04678

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-45
because they relate to subject matter not required to be searched by this Authority, namely:
See FURTHER INFORMATION SHEET PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 1-45 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

Claims Nos.: 1-45

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ational Application No
PCT/US 99/04678

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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